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A novel method of identifying binding site domains that retain the capacity of binding to an epitope

The present invention relates to a method of identifying domains having binding affinity for a preselected epitope. The domains comprise preferably immunoglobulin V_H and V_L domains that retain the capacity of binding to an epitope when positioned C-terminal of at least one further domain in a recombinant bi- or multivalent polypeptide. The present invention further relates to a kit comprising components such as panels of recombinant vectors or bacterial libraries transfected with a panel of recombinant vectors which is useful in carrying out the method of the invention. Furthermore, the present invention relates to polypeptides obtainable by the afore-described method and their use in pharmaceutical and diagnostic compositions.

Multivalent receptors such as recombinant bifunctional antibody constructs play an increasingly important therapeutic and scientific role in particular in the medical field, for example, in the development of new treatment approaches for cancer and autoimmune diseases or as interesting tools for the analysis and modulation of cellular signal transduction pathways, pioneer work has been done using such receptors.

Thus, by cross-linking of the CD3-activation antigen on T cells with a tumor associated antigen on tumor cells, bispecific single-chain antibodies can bring both cells together so that the tumor cell is efficiently lysed during the cell-cell contact (Mack, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 7021-7025). Comparable approaches have been or are being developed for other target cells (e.g. virus-infected cells) and for the recruitment of other effector cell populations (e.g. NK-cells and mononuclear phagocytes). Using bifunctional

fusion proteins that carry an antibody fragment as targeting mechanism, a large number of different receptors and ligands can be specifically bound to defined surface molecules on selected cell populations. It is particularly interesting that surface molecules on the same cell can be cross-linked by bi-specific antibodies in order to modulate cellular function or the state of activation or differentiation of such cells. A possible application of this type of approach may be the induction of anergy in auto-aggressive B- or T-lymphocytes that play a pathogenetic role in many autoimmune diseases. Regarding the broad scientific and therapeutic relevance, efficient and reproducible methods for producing recombinant polypeptides comprising functional antigen binding sites are of particular importance; such methods yield, for example, functionally active bispecific antibody constructs by expression in bacteria and in mammalian cells. Said recombinant bifunctional single-chain proteins usually are built up by different scFv-antibody fragments, each of which consists of one immunoglobulin variable heavy (V_H) and one variable light (V_L)-antigen binding domain. Alternatively, they may comprise such an antibody fragment and one non-immunoglobulin part. All functional domains are located on a single polypeptide chain and joined together by flexible Glycin-Serin- or other appropriate peptide linkers. The bifunctional polypeptide chain can be produced as functional protein by transfecting mammalian or less preferentially other host cells with the corresponding DNA-sequence, that additionally may encode an optional protein-tag, preferentially a poly-histidine-tag, enabling easy purification of the recombinant protein for example by using a nickel-chelate-column. The production of multivalent and preferably bifunctional constructs according to this single-chain approach has important advantages compared to conventional methods using in vitro- or in vivo-heterodi- or multimerization of independently expressed functional domains, a procedure that can be very laborious and frequently associated with low yields. The appearance of contaminating homodimers is excluded by the single-chain approach, thus resulting in protein preparations of high purity and yield since all the recombinant protein produced consists to 100% of the desired bifunctional

construct. As has been demonstrated by way of example with a bispecific single-chain antibody functionally expressed in CHO-cells, scFv-antibody fragments can in principle bind to their antigen either as the N-terminal or the C-terminal part of a bifunctional single-chain construct, (Mack, Proc. Natl. Acad. Sci. U.S.A. 92(1995) 7021-7025).

However, many functional domains of multivalent polypeptides such as antibody fragments lose their binding activity when located C-terminal of a further three-dimensional proteinaceous structure within a fusion protein. For example, scFv-fragments derived from randomly selected antibodies produced by hybridoma cell lines or selected in vitro from combinatorial antibody libraries frequently lose their antigen binding activity when located at the C-terminal position within recombinant bifunctional single-chain proteins, although the same V_H/V_L-pairs bind to the antigen when located at the N-terminus or as whole antibodies or free monovalent scFv-fragments (Figure 10). This phenomenon was, by way of reference Examples, extensively characterized with recombinant bifunctional single-chain molecules consisting at the N-terminus of the extra-cellular part of human CD80 (B7-1) followed at the C-terminus by different scFv-fragments derived form antibodies that specifically bind to the 17-1A-antigen (Figure 1.1). Of four different 17-1A-specific antibodies, three of which were produced by murine hybridoma cell lines and one selected in vitro from a human combinatorial antibody library using the phage display method, none gave raise to a scFv-fragment that retains its antigen binding activity when fused with its N-terminus to the C-terminus of the human CD80-fragment and expressed as bifunctional single-chain molecule in CHO-cells (Examples 1-4). It is noteworthy that two of the murine antibody fragments (M79 and M74) bind to the 17-1A-antigen as N-terminal part of bi-specific single-chain antibodies (Mack, Proc. Natl. Acad. Sci. U.S.A. (1995) 7021-7025) as well as in the form of free monovalent scFv-fragments, the latter of which was also shown for the human 17-1A-specific antibody VD4.5VK8 (Example 3) derived in vitro from a phage library. All four specificities bind to the 17-1A-antigen in the form of whole antibody

molecules. Accordingly, the technical problem underlying the present invention was to provide means and methods to identify bi- or multivalent polypeptides that comprise antibody binding sites capable of efficiently binding to the corresponding antigen. The solution to said technical problem is achieved by providing the embodiments characterized in the claims.

Thus, the present invention relates to a method of identifying a binding site domain having the capacity of binding to a predetermined epitope when positioned C-terminal of at least one further domain in a recombinant bi- or multivalent polypeptide comprising the steps of

(a) testing a panel of binding site domains displayed on the surface of a biological display system as part of a fusion protein for binding to a predetermined epitope, wherein said fusion protein comprises an additional domain positioned N-terminal of said binding site domain and an amino acid sequence that mediates anchoring of the fusion protein to the surface of said display system; and

(b) identifying a binding site domain that binds to said predetermined epitope.

Preferably, the binding site domain capable of binding to a preselected antigenic determinant comprises an amino acid sequence homologous with the sequence of a variable region of an immunoglobulin molecule capable of binding said preselected epitope.

The term "binding site domain" as used in accordance with the present invention denotes a domain comprising a three-dimensional structure capable of binding to an epitope.

The term "bi- or multivalent polypeptide" as used herein denotes a polypeptide comprising at least two amino acid sequences derived from different origins wherein one of said origins specifies the binding site domain.

In accordance with the present invention, the term "capacity of binding to an epitope" denotes the capacity of said binding site domain to enter and bind a corresponding epitope, like native antibodies or free scFv fragments.
The term "panel" as used in accordance with the present invention relates to two or more pairs of the recited domains. Preferably, said panel is derived from a library such as a cDNA library, or, more preferably, a combinatorial library of, e.g., V_H/V_L chains.

The fusion protein is capable of binding to a preselected epitope and preferably, has a specificity at least substantially identical to the binding specificity of the, e.g., immunoglobulin molecule where it is derived from. Such binding site domains can have a binding affinity of at least 10^6M^{-1}, preferably 10^8M^{-1} and advantageously up to $10^{10}M^{-1}$ or higher.
The additional domain present in the fusion protein may be linked by a polypeptide linker to the binding site domain. Furthermore, said additional domain may be of a predefined specificity or function. For example, the literature contains a host of references to the concept of targeting bioactive substances such as drugs, toxins, and enzymes to specific points in the body to destroy or locate malignant cells or to induce a localized drug or enzymatic effect. It has been proposed to achieve this effect by conjugating the bioactive substance to monoclonal antibodies (see, e.g., N.Y. Oxford University Press; and Ghose, (1978) J. Natl. Cancer Inst. 61:657-676). However, constructing corresponding targeted multifunctional proteins is hampered by the fact that the chimeric proteins loose their binding affinity and/or specificity due to the presence of extra sequences and guess work turned out to be insufficient to remedy this obstacle.
The method of the present invention can solve this problem and thus can be used to prepare and identify such multi-functional proteins which substantially retain both, the binding affinity and the function of the additional domain(s).
In a preferred embodiment of the method of the invention the binding site domain and said additional domain are linked by a polypeptide linker disposed between said binding site and said additional domain, wherein said

polypeptide linker comprises plural, hydrophilic, peptide-bonded amino acids and connects the N-terminal end of said binding site and the C-terminal end of said additional domain.

As well known, Fv, the minimum antibody fragment which contains a complete antigen recognition and binding site, consists of a dimer of one heavy and one light chain variable domain (V_H and V_L) in noncovalent association. It is in this configuration that the three complementarity determining regions (CDRs) of each variable domain interact to define an antigen binding site on the surface of the V_H-V_L dimer. Collectively, the six CDRs confer antigen binding specificity to the antibody. Frameworks (FRs) flanking the CDRs have a tertiary structure which is essentially conserved in native immunoglobulins of species as diverse as human and mouse. These FRs serve to hold the CDRs in their appropriate orientation. The constant domains are not required for binding function, but may aid in stabilizing V_H-V_L interaction. Even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than an entire binding site (Painter (1972) Biochem. 11:1327-1337).

Hence, in a particularly preferred embodiment of the method of the invention, said binding site domain is a pair of V_H-V_L, V_H-V_H or V_L-V_L domains either of the same or of different immunoglobulins.

The order of V_H and V_L domains within the polypeptide chain is not decisive for the present invention, the order of domains given herein above may be reversed without any loss of function. It is important, however, that the V_H and V_L domains are arranged so that the antigen binding site can properly fold.

In accordance with the present invention, the term "identify" relates, in its broadest sense, to the identification of a clone that comprises the properly binding site domain, preferably said clone can be purified and the sequence of the binding site domain, e.g., V_H and V_L domains may be determined.

Naturally, the method of the invention is not only applicable to the identification of a single pair of V_H and V_L domains, but may also be applied to the identification and isolation of a variety of such pairs.

Prior to establishing the method of the invention, a variety of parameters were considered that were expected to possibly influence the binding activity of scFv-antibody fragments located at the C-terminus of multivalent polypeptides, in particular of bifunctional single-chain molecules. Thus, constructs with 5- and 15-amino acid glycin-serin-linkers between the CD80- and the scFv-fragment as well as alternative domain arrangements, namely V_L-V_H and V_H-V_L within the C-terminal scFv-fragment were produced and analysed for antigen binding (Examples 1 and 2). However, antigen binding of scFv-fragments that lost their binding activity due to their position at the C-terminus of bifunctional single-chain molecules could not be reconstituted by using different linker lengths and/or by changing the arrangement of the V_L- and the V_H-domains in any Example tested.
Surprisingly, it was now found in accordance with the present invention that by using a novel in vitro selection method based on the phage display technology (Figure 11), scFv-antibody fragments that bind independently of their position within bifunctional single-chain fusion proteins could be isolated from, by way of Example, combinatorial antibody libraries, (Examples 5 and 6).

The present invention thus significantly extends the applicability of multivalent polypeptides such as bifunctional single-chain molecules.

To functionally simulate the C-terminal context in multivalent polypeptides exemplified by bifunctional single-chain constructs, the N-terminus of V_H-V_L-scFv-antibody fragments, respectively that of the V_H-domain, was fused to the C-terminus of a stretch of amino acids folding into a three-dimensional structure. Experimentally, this was achieved by employing the N2-domain of the gene III-product of filamentous phage (Krebber, FEBS Letters 377 (1995)

227-231). Accordingly, the N2-domain plays the role of a surrogate for any preferably functional domain located at the N-terminus of a pair of V_H and V_L domains within a bi- or multivalent single-chain protein. The "N-terminally blocked" scFv-fragment N2-V_H-V_L, respectively the C-terminus of V_L, was fused to an amino acid sequence that mediates anchoring of the fusion protein to the surface of a phage. Experimentally, this was effected by employing the N-terminus of the C-terminal CT-domain of the gene III-filamentous phage product (Barbas, Proc. Natl. Acad. Sci. U.S.A. 88 (1991) 7978-7982). In the following, the invention will be explained in more detail on the basis of the experiments that were actually carried out: The DNA encoding the fusion protein N2-V_H-V_L-CT can be cloned into a phagemid vector (e.g. pComb3H) and transformed into a male E.coli-strain (e.g. XL1-blue) that will, after infection with a filamentous helper phage, produce phage particles carrying the N2-V_H-V_L-CT-fusion protein on their surface and containing a single-stranded copy of the corresponding DNA. This coupling of phenotype and genotype enables to select and enrich - by several rounds of panning on the antigen - from large repertoires of V_H/V_L-combinations those "N-terminally blocked" scFv-antibody fragments that nevertheless retain their antigen binding activity. To test the method of the invention, mice were immunized with recombinant soluble 17-1A-antigen; animals with detectable anti-17-1A serum antibody titer were sacrificed, total RNA was prepared from the murine spleen cells and reverse-transcribed into cDNA using random hexamer priming. The V_L- and V_H-repertoire of the current antibody response was amplified by PCR using V_L- and V_H-subfamily specific oligonucleotide primers and cloned into the phagemid vector pComb3H already containing the DNA-sequences encoding the N2- and the CT-domain of the gene III-product of filamentous phage. This combinatorial antibody library was transformed into the E.coli-strain XL1-blue to subsequently proceed with the in vitro-selection by panning on immobilized 17-1A-antigen according to the phage display method (Winter, Annu. Rev. Immunol. 12 (1994) 433-455; Barbas, METHODS, A companion to Methods in Enzymology 2 (1991) 119-124). After the third, fourth and fifth round of panning, soluble N2-V_H-V_L-single

chain fragments of individual clones were generated by the excision of the gene III-CT-sequence prior to the periplasmatic expression in E.coli and tested by ELISA for binding to immobilized 17-1A-antigen. The V_L- and V_H-regions of "N2-blocked" scFv-fragments that bound to the 17-1A-antigen were sequenced and subcloned into the mammalian expression vector pEF-DHFR already containing the coding sequence of the extra-cellular CD80-fragment thus resulting in a final construct that encodes a bifunctional single-chain protein with the CD80-fragment located at the N-terminal position (Example 7). In addition, one V_H-V_L-pair derived from a 17-1A-specific murine hybridoma cell line (Example 4) and another 17-1A-specific V_H-V_L-pair selected from a human combinatorial antibody library by the conventional phage display method (Example 3) were also cloned into this bifunctional context. The bifunctional single-chain constructs were transfected into DHFR-deficient CHO-cells using nucleoside-free culture medium for the primary selection and the protein expression was subsequently increased by gene amplification using the DHFR-inhibitor methotrexat at a final concentration of 20nM. The recombinant bifunctional proteins were secreted into the culture supernatant; the culture supernatants from the different clones were analysed for antigen binding by ELISA on immobilized recombinant 17-1A-antigen (Example 8) and by flow cytometry on CHO-cells transfected with the transmembrane form of the 17-1A-antigen (Example 9). All of the nine different bifunctional single-chain constructs derived from the method of the invention proved to bind to the 17-1A-antigen as demonstrated in both binding assays (ELISA and FACS) (Figures 8.1, 8.2 and 9.1); both conventionally derived bifunctional single-chain constructs, however, failed to bind to the 17-1A-antigen (Figures 8.3, 8.4 and 9.1). Taken together, these data demonstrate that scFv-antibody fragments that retain their antigen binding activity at the C-terminal position of bifunctional single-chain proteins can be selectively obtained by the method of the invention involving an N-terminal surrogate domain simulating the effect of other functional domains fused to the N-terminus of scFv-antibody fragments. This exemplary approach can, by the person skilled in the art, be transferred to any other pair

of V_H and V_L domains comprised in a multivalent polypeptide in the above indicated position(s).

In a preferred embodiment of the present invention, said biological display system is filamentous phage produced by bacteria transfected therewith, a baculovirus expression system, a ribosome based display system, a bacteriophage lambda display system or a bacterial surface expression system based, for example, on the ompA protein.

An Example of a ribosome display system has been described, for example, by Hanes, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 4937-4942. Examples of the other systems referred to above are well established in the art (Mottershead, Biochem. Biophys. Res. Commun. 238 (1997) 717-722; Sternberg, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 1609-1613; Stahl, Trends Biotechnol, 15 (1997) 185-192).

As regards the bacteria transfected with the phage, it is preferred that the bacteria are E.coli.

Referring now to the experimental procedure used to explain the invention and described herein above, in a further preferred embodiment of the invention, said method comprises prior to step (a), the further step of (a") transfecting bacteria with recombinant vectors encoding said fusion proteins. Preferably, said vectors are phagemid vectors.

In a further preferred embodiment of the invention, said method comprises prior to step (a"), the further step of (a') cloning a panel of nucleic acid molecules encoding the binding site domain, e.g., pairs of V_H and V_L domains into a vector.

In a most preferred embodiment of the invention, said panel of nucleic acid molecules is derived from immune competent cells of a mammal, fish or bird.

This embodiment is particularly preferred insofar as it reflects the immune repertoire of the B-cell compartment of the mammal which may be amplified and cloned by RT-PCR using V_L- and V_H-specific oligonucleotide primers or primer sets.

In an additional preferred embodiment of the invention, said additional domain comprises at least 9 amino acids.
Preferably, said additional domain is not sufficient to mediate phage infectivity when displayed on the surface of phage particles.

In a most preferred embodiment of the invention, said additional domain is or is derived from the N2-domain of the gene III product of filamentous phage. Preferably, N2 is not capable of mediating infectivity of the phage.

In a preferred embodiment of the invention, said sequence mediating said anchoring is or is derived from the C-terminal CT-domain of the gene III product of filamentous phage. However, other suitable domains known to be capable of mediating anchoring to surfaces of, e.g., phage displays may be used as well.

In a further preferred embodiment of the invention, said bi- or multivalent polypeptide is a bi- or multifunctional polypeptide.

In a most preferred embodiment of the invention, said at least one further domain comprises a polypeptide selected from the group consisting of effector proteins having a conformation suitable for biological activity, amino acid sequences capable of sequestering an ion, and amino acid sequences capable of selective binding to a solid support.
Preferably, said effector protein is an enzyme, toxin, receptor, binding site, biosynthetic antibody binding site, growth factor, cell-differentiation factor, lymphokine, cytokine, hormone, a remotely detectable moiety, or anti-metabolite.

Furthermore, said sequence capable of sequestering an ion is preferably selected from calmodulin, methallothionein, a fragment thereof, or an amino acid sequence rich in at least one of glutamic acid, aspartic acid, lysine, and arginine.

In addition, said polypeptide sequence capable of selective binding to a solid support can be a positively or negatively charged amino acid sequence, a cysteine-containing amino acid sequence, avidin, streptavidin, or a fragment of Staphylococcus protein A.

The effector proteins and amino acid sequences described above may be present in a proform which itself is either active or not and which may be removed, when, e.g., entering a certain cellular environment.

In a most preferred embodiment of the invention, said receptor is a costimulatory surface molecule important for T-cell activation or comprises an epitope binding site or a hormone binding site.

In a further most preferred embodiment of the invention, said costimulatory surface molecule is CD80 (B7-1).

In a further most preferred embodiment of the invention, said epitope binding site is embedded in a pair of V_H-V_L, V_H-V_H and V_L-V_L domains.

In a preferred embodiment of the invention, said V_H and/or V_L domains are connected by a flexible linker, preferably by a polypeptide linker disposed between said domains, wherein said polypeptide linker comprises plural, hydrophilic, peptide-bonded amino acids of a length sufficient to span the distance between the C-terminal end of one of said domains and the N-terminal end of the other of said domains when said fusion protein assumes a conformation suitable for binding when disposed in aqueous solution.

In a further preferred embodiment of the invention, the identification of said binding site domain comprises the steps of

(a) removing said amino acid sequence that mediates anchoring of the fusion protein to the surface of a phage from said fusion protein;

(b) periplasmatically expressing the nucleic acid molecules encoding the remainder of said fusion protein in bacteria; and

(c) verifying whether said binding site domain binds to said predetermined epitope.

In another embodiment the present invention relates to a recombinant vector as defined in the above-described embodiments and to a host cell harboring and capable of expressing such a recombinant vector.

In a further preferred embodiment of the invention, the kit comprises

(a) the described recombinant vector or a panel of recombinant vectors encoding a panel of fusion proteins as defined in the embodiments described above; and/or

(b) the described host cell or a bacterial library transfected with a panel of vectors as defined in (a).

Furthermore, the present invention relates to a binding site domain or fusion protein obtainable by the method of the invention as characterized in the embodiments above. Advantageously, the amino acid sequence that mediates anchoring of the fusion protein to the surface of a phage of said fusion protein is removed from the fusion protein. Thus, the resultant fusion protein may only comprise the binding site domain and an additional domain, preferably an effector protein as described above.

In a preferred embodiment of the present invention, the binding site domain, for example contained in a fusion protein comprises at least one complementarity determined region (CDR) of the scFv fragment shown in any one of Figures 6.3 to 6.10 and 7. The person skilled in the art knew that each

variable domain comprises three hypervariable regions, sometimes called complementarity determining regions or "CDRs" flanked by four relatively conserved framework regions or "FRs". The CDRs contained in the variable regions shown in Figures 6.3 to 6.10 and 7 can be determined according to Kabat, Sequences of Proteins of Immunological Interest (U.S. Department of Health and Human Services, third edition, 1983, fourth edition, 1987, fifth edition 1990).

The person skilled in the art will readily appreciate that the binding site domain or fusion protein identified according to the method of the invention or at least one CDR derived therefrom can be used for the construction of other polypeptides or antibodies of desired specificity and biological function. Thus, the present invention also relates to polypeptides and antibodies comprising a binding site domain or fusion protein of the invention. Preferably, said polypeptide or antibody comprises the amino acid sequence as depicted in any one of Figures 6.3 to 6.10 and 7. The person skilled in the art will readily appreciate that using the binding sites or CDRs described above antibodies can be constructed according to methods known in the art, e.g., as described in EP-A1 0 451 216 and EP-A1 0 549 581.

Yet in a further embodiment, the present invention relates to polynucleotides which upon expression encode the above-described polypeptides and antibodies. Said polynucleotides may be fused to suitable expression control sequences known in the art to ensure proper transcription and translation of the polypeptide. Furthermore, the polynucleotides may be comprised in a vector which further comprises a selectable marker.

In a still further embodiment, the present invention relates to a cell containing the polynucleotide described above. Preferably, said cell is a mammalian cell if therapeutic uses of the polypeptide are envisaged. Of course, yeast and bacterial cells may serve as well, in particular if the produced polypeptide is used as a diagnostic means.

In a further embodiment, the present invention thus relates to a process for the preparation of a fusion protein obtainable by the method according to the invention, a polypeptide or antibody as described above comprising cultivating a cell of the invention under conditions suitable for the expression of the fusion protein or polypeptide and isolating the fusion protein, polypeptide or antibody from the cell culture medium.

Moreover, the present invention relates to a pharmaceutical composition containing a fusion protein, polypeptide or antibody of the invention and optionally a pharmaceutically acceptable carrier.

As to a further embodiment, the present invention relates to a diagnostic composition comprising a fusion protein, polypeptide or antibody as described above and optionally suitable means for detection.

The present invention allows recombinant production of single chain binding sites having affinity and specificity for a predetermined epitope. This technology has been developed and is disclosed herein. In view of this disclosure, persons skilled in recombinant DNA technology, protein design, and protein chemistry can produce such sites which, when disposed in solution, have high binding constants (usually at least 10^6, preferably $10^8 M^{-1}$) and excellent specificity. As is evident from the foregoing, the invention provides a large family of binding site domains and fusion proteins as well as polypeptides comprising such binding site domains and fusion proteins for any use in therapeutic and diagnostic approaches. It will be apparent to those skilled in the art that the binding site domains and fusion proteins can be further coupled to other moieties for, e.g., drug targeting and imaging applications. Such coupling may be conducted chemically after expression of the fusion proteins or polypeptides to site of attachment or the coupling product may be engineered into the polypeptide of the invention at the DNA level. The DNAs are then expressed in a suitable host system, and the expressed proteins are collected and renatured, if necessary. As described

above, the binding site domain is preferably derived from the variable region of antibodies, preferably monoclonal antibodies. In this respect, hybridoma technology enables production of cell lines secreting antibody to essentially any desired substance that produces an immune response. RNA encoding the light and heavy chains of the immunoglobulin can then be obtained from the cytoplasm of the hybridoma. The 5' end portion of the mRNA can be used to prepare cDNA to be used in the method of the present invention.

The DNA encoding the fusion proteins obtained according to the method of the invention can then be expressed in cells, preferably mammalian cells.

Depending on the host cell, renaturation techniques may be required to attain proper conformation. The various proteins can then be further tested for binding ability, and one having appropriate affinity can be selected for incorporation into a polypeptide of the type described above. If necessary, point substitutions seeking to optimize binding may be made in the DNA using conventional cassette mutagenesis or other protein engineering methodology such as is disclosed below.

Preparation of the polypeptides of the invention also is dependent on knowledge of the amino acid sequence (or corresponding DNA or RNA sequence) of bioactive proteins such as enzymes, toxins, growth factors, cell differentiation factors, receptors, anti-metabolites, hormones or various cytokines or lymphokines. Such sequences are reported in the literature and available through computerized data banks.

The DNA sequences of the binding site and the second protein domain are fused using conventional techniques, or assembled from synthesized oligonucleotides, and then expressed using equally conventional techniques.

The processes for manipulating, amplifying, and recombining DNA which encode amino acid sequences of interest are generally well known in the art,

and therefore, not described in detail herein. Methods of identifying and isolating genes encoding antibodies of interest are well understood, and described in the application and other literature. In general, the methods involve selecting genetic material coding for amino acids which define the proteins of interest, including the CDRs and FRs of interest, according to the genetic code.

Accordingly, the construction of DNAs encoding proteins as disclosed herein can be done using known techniques involving the use of various restriction enzymes which make sequence specific cuts in DNA to produce blunt ends or cohesive ends, DNA ligases, techniques enabling enzymatic addition of sticky ends to blunt-ended DNA, construction of synthetic DNAs by assembly of short or medium length oligonucleotides, cDNA synthesis techniques, and synthetic probes for isolating immunoglobulin or other bioactive protein genes. Various promoter sequences and other regulatory DNA sequences used in achieving expression, and various types of host cells are also known and available. Conventional transfection techniques, and equally conventional techniques for cloning and subcloning DNA are useful in the practice of this invention and known to those skilled in the art. Various types of vectors may be used such as plasmids and viruses including animal viruses and bacteriophages. The vectors may exploit various marker genes which impart to a successfully transfected cell a detectable phenotypic property that can be used to identify which of a family of clones has successfully incorporated the recombinant DNA of the vector.

These and other embodiments are disclosed and encompassed by the description and Examples of the present invention. For example, further literature concerning any one of the methods, uses and compounds to be employed in accordance with the present invention may be retrieved from public libraries, using for example electronic devices. For example the public database "Medline" may be utilized which is available on the Internet, for example under http://www.ncbi.nlm.nih.gov/PubMed/medline.html. Further

databases and addresses, such as http://www.ncbi.nlm.nih.gov/, http://www.infobiogen.fr/, http://www.fmi.ch/biology/research_tools.html, http://www.tigr.org/, are known to the person skilled in the art and can also be obtained using, e.g., http://www.lycos.com. An overview of patent information in biotechnology and a survey of relevant sources of patent information useful for retrospective searching and for current awareness is given in Berks, TIBTECH 12 (1994), 352-364.

The figures show:

Figure 1.1: Design of various bifunctional CD80-scFv-constructs showing the construction elements on the protein-level. V_H indicates the variable region of the Ig-heavy chain, V_L that of the Ig-light chain. The single-chain-Fv-fragments used in the present invention are given in the Examples 1, 2, 3, 4 and 9.

Figure 1.2: DNA sequence designated CTI that was cloned into the multiple cloning site of the Bluescript KS vector (GenBank® accession number X52327) by using the restriction sites XbaI and SalI in order to increase the number of possible cloning sites. CTI-derived restriction enzyme cleavage sites are shown.

Figure 1.3: Design of various bifunctional CD80-scFv-constructs showing the construction elements on the DNA-level as well as the restriction enzyme cleavage sites used.

Figure 1.4: ELISA-analysis of the cell-culture supernatant obtained from CHO cells transfected with the expression plasmid pEF-DHFR+CTI+CD80-M79scFv(V_L/V_H) including the coding sequence of the short $(Gly_4Ser_1)_1$ linker. 96 well ELISA plates were incubated with 50µl of soluble 17-1A antigen (50µg/ml) per well. Subsequently pure cell-culture supernatant dilutions thereof were added as indicated. Detection was performed by a murine IgG1

anti His-tag antibody (dianova, Hamburg) diluted 1:200 and a peroxidase conjugated polyclonal goat anti mouse IgG (Fc) antibody (dianova, Hamburg) diluted 1:5000. The anti-17-1A/anti-CD3 bispecific-single-chain antibody (Mack, Proc. Natl. Acad. Sci. USA 92 (1995) 7021-7025) was used as positive control. As negative control, wells were incubated with phosphate buffered saline. The ELISA was developed by ABTS -substrate solution as described in Example 8. The OD-values were measured at 405nm by an ELISA reader.

Figure 1.5: ELISA-analysis of the cell-culture supernatant obtained from CHO-cells transfected with the expression plasmid pEF-DHFR+CTI+CD80-M79scFv(V_L/V_H) including the coding sequence of the short $(Gly_4Ser_1)_1$ linker. 96 well ELISA plates were incubated with 50µl soluble 17-1A antigen (50µg/ml) per well. Subsequently pure cell-culture supernatant and dilutions thereof were added as indicated. Detection was performed by a murine IgG1-anti CD80 antibody diluted 1:1000 followed by a peroxidase conjugated polyclonal goat anti mouse IgG (Fc) antibody (dianova, Hamburg) diluted 1:5000. The anti-17-1A/anti-CD3 bispecific-single-chain antibody (Mack, Proc. Natl. Acad. Sci. USA 92 (1995) 7021-7025). was used as positive control and detected as described in Figure 1.4. As negative control, wells were incubated with phosphate buffered saline. The ELISA was developed by an ABTS -substrate solution as described in Example 8. The OD-values were measured at 405nm by an ELISA reader.

Figure 1.6: ELISA-analysis of the purified recombinant CD80-M79scFv(V_L/V_H)-construct with a short $(Gly_4Ser_1)_1$ linker obtained by purification from cell-culture supernatant using a Ni-NTA-column as described (Mack, Proc. Natl. Acad. Sci. USA 92 (1995) 7021-7025). 96 well ELISA plates were coated overnight at 4°C with pure eluate from the Ni-NTA-column and dilutions thereof as indicated. Subsequently bound recombinant protein was detected by a murine IgG1-anti CD80 antibody diluted 1:1000 or by a murine IgG1-anti His-tag antibody (dianova, Hamburg) diluted 1:200 followed

by a peroxidase conjugated polyclonal goat anti mouse IgG (Fc) antibody (dianova, Hamburg) respectively diluted 1:5000. As negative control wells were coated overnight at 4°C with 3% BSA in phosphate buffered saline. The ELISA was developed by an ABTS -substrate solution as described in Example 8. The OD-values were measured at 405nm by an ELISA reader.

Figure 1.7: ELISA-analysis of the cell-culture supernatant obtained from CHO-cells transfected with the expression plasmid pEF-DHFR+CTI+CD80-M79scFv(V_H/V_L) including the coding sequence of the short $(Gly_4Ser_1)_1$ linker. 96 well ELISA plates were incubated with soluble 17-1A antigen (50µg/ml) per well. Subsequently pure cell-culture supernatant and dilutions: thereof were added as indicated. Detection was performed by a murine IgG1-anti CD80 antibody diluted 1:1000 followed by a peroxidase conjugated polyclonal goat anti-mouse IgG (Fc) antibody (dianova, Hamburg) diluted 1:5000. The anti-17-1A/anti-CD3 bispecific-single-chain antibody (Mack, Proc. Natl. Acad. Sci. USA 92 (1995) 7021-7025) was used as positive control and detected as described in Figure 1.4. As negative control wells were incubated with phosphat buffered saline. The ELISA was processed by an ABTS-substrate solution as described in Example 8. The OD-values were measured at 405nm by an ELISA reader.

Figure 1.8: DNA-sequence of the double-stranded oligonucleotide designated ACCGS15BAM with single-stranded overhangs compatible with those of restriction enzymes BspEI and BamHI. Amino acids encoded by the nucleotide sequence are shown.

Figure 1.9: ELISA-analysis of the cell-culture supernatant and of its dilutions obtained from CHO-cells transfected with the expression plasmid pEF-DHFR+CTI+CD80-M79scFv (V_H/V_L) including the coding sequence of the long $(Gly_4Ser_1)_3$ linker. 96 well ELISA plates were incubated with 50µl soluble 17-1A antigen (50µg/ml) per well. Subsequently pure cell-culture supernatant and dilutions thereof were added as indicated. Bound protein was detected by

a murine anti His-tag antibody (dianova, Hamburg) diluted 1:200 followed by a peroxidase conjugated polyclonal goat anti mouse IgG (Fc) antibody (dianova, Hamburg) diluted 1:5000. The anti-17-1A/anti-CD3 bispecific-single-chain antibody antibody (Mack, Proc. Natl. Acad. Sci. USA 92 (1995) 7021-7025) was used as positive control. As negative control wells were incubated with phosphat buffered saline. The ELISA was developed by an ABTS-substrate solution as described in Example 8. The OD-values were measured at 405nm by an ELISA reader.

Figure 2.1: ELISA-analysis of the cell-culture supernatant obtained from CHO-cells transfected with the expression plasmids pEF-DHFR+CTI+CD80-M74scFv(V_H/V_L) or pEF-DHFR + CTI + CD80-M74scFv(V_L/V_H) including the coding sequence of the long $(Gly_4Ser_1)_3$ or short $(Gly_4Ser_1)_1$ linker respectively. 96 well ELISA plates were incubated with 50µl soluble 17-1A antigen (50µg/ml) per well. Subsequently pure cell-culture supernatant and dilutions thereof were added as indicated. Detection was performed by a murine IgG1 anti His-tag antibody (dianova, Hamburg) diluted 1:1000 and followed by a peroxidase conjugated polyclonal goat anti mouse IgG (Fc) antibody (dianova, Hamburg) diluted 1:5000. The anti-17-1A/antiCD3 bispecific-single-chain antibody antibody (Mack, Proc. Natl. Acad. Sci. USA 92 (1995) 7021-7025) was used as positive control and detected as described in Figure 1.4. As negative control wells were incubated with phosphat buffered saline. The ELISA was developed by an ABTS-substrate solution as described in Example 8. The OD-values were measured at 405nm by an ELISA reader.

Figure 2.2: ELISA-analysis of the cell-culture supernatant obtained from CHO-cells transfected with the expression plasmids pEF-DHFR+CTI+CD80-M74scFv(V_H/V_L) or pEF-DHFR + CTI + CD80-M74scFv(V_L/V_H) including the coding sequence of the long $(Gly_4Ser_1)_3$ or short $(Gly_4Ser_1)_1$ linker respectively. 96 well ELISA plates were incubated with 50µl soluble 17-1A antigen (50µg/ml) per well. Subsequently pure cell-culture supernatant and

dilutions thereof were added as indicated. Detection was performed by a murine IgG1-anti CD80 antibody diluted 1:1000 followed by a peroxidase conjugated polyclonal goat anti mouse IgG (Fc) antibody (dianova, Hamburg) diluted 1:5000. The anti-17-1A/anti-CD3 bispecific-single-chain antibody antibody (Mack, Proc. Natl. Acad. Sci. USA 92 (1995) 7021-7025) was used as positive control and detected as described in Figure1.4. As negative control wells were incubated with phosphat buffered saline. The ELISA was developed by an ABTS -substrate solution as described in Example 8. The OD-values were measured at 405nm by an ELISA reader.

Figure 3.1: DNA- and protein sequence of the human D4.5. heavy chain variable region (V_H of the human anti-17-1A-antibody VD4.5VK8). Number indicate the nucleotide (nt) positions, amino acids are presented in the single letter code. CDR1 includes nt 91 to nt 105, CDR2 nt 148 to nt 198, CDR3 nt 292 to nt 351. The border between the heavy chain variable region and the CH1 domain is located between nt 382 and nt 383.

Figure 3.2: DNA- and protein sequence of the human kappa 8 light chain variable region (V_L of the human anti-17-1A-antibody VD4.5VK8). Numbers indicate the nucleotide (nt) positions, amino acids are presented in single letter code. CDR1 includes nt 70 to nt 102, CDR2 nt 148 to nt 168, CDR3 nt 265 to nt 294.

Figure 3.3: ELISA-analysis of free scFv-fragment (V_H/V_L) of the human anti 17-1A antibody VD4.5VK8. The sequence encoding the N2-domain was excised from the plasmid pComb3H5BHis-VD4.5VK8scFv (Example 3) using the restriction enzymes SalI and XhoI followed by religation of the vector. The resulting plasmid was used for periplasmatic expression of soluble VD4.5VK8-scFv-fragment in E.coli XL1-blue according to the procedure described in Example 6. Analysis of binding to the 17-1A-antigen of soluble VD4.5VK8-scFv-fragment was performed as follows: 96 well ELISA plates were incubated with soluble 17-1A antigen (50µg/ml). Subsequently, pure periplasma preparation was added. Detection was performed by a murine

IgG1-anti-His-tag antibody diluted 1:250 followed by a peroxidase conjugated polyclonal goat anti mouse IgG (Fc) antibody (dianova, Hamburg) diluted 1:5000. The anti-17-1A/anti-CD3 bispecific-single-chain antibody (Mack, Proc. Natl. Acad. Sci. USA 92 (1995) 7021-7025) was used as positive control and detected as described in Figure 1.4. As negative control, an irrelevant periplasma preparation was used. The ELISA was developed by an ABTS - substrate solution as described in Example 8. The OD-values were measured at 405nm by an ELISA reader.

Figure 3.4: NS3 Frame: DNA-sequence designated L-F-NS3Frame that was cloned into the multicloning site of the vector Bluescript-KS-CTI (Figure1.2) by using the restriction sites EcoRI and SalI in order to increase the number of possible cloning sites. Cloning sites derived from L-F-NS3Frame are shown.

Figure 4: ELISA-analysis of the cell-culture supernatant obtained from CHO-cells transfected with the light and heavy chain of the chimerized anti 17-1A antibody MACH (Example 4). 96 well ELISA plates were incubated with soluble 17-1A antigen (50µg/ml). Subsequently, pure cell-culture supernatant and dilutions thereof were added as indicated. Detection was performed by a biotinylated anti human IgG antibody followed by streptavidin. Supernatant of the parent murine anti-17-1A antibody MACH and dilutions thereof were used as positive control and detected by a biotiylated anti-mouse IgG antibody. As negative control, phosphat buffered saline was used. The ELISA was developed by an ABTS -substrate solution as described in Example 8. The OD-values were measured at 405nm by an ELISA reader.

Figure 5.1: Cloning site of pComb3H with important restriction sites. The following abbreviations were used: P, lac-promotor; V_L, variable light chain domain; CL, constant light chain domain; V_H, variable heavy chain domain; CH1, constant heavy chain domain; L1/2, procaryotic leader sequences (L1 = ompA, L2 = pelB).

Figure 5.2: DNA sequence of the multiple cloning site of pComb3H5BHis showing important restriction enzyme cleavage sites as well as the amino acid sequence of the Glycine-Serine-linker and that of the N2-domain of the gene III-product of filamentous phage.
The DNA-sequence encoding the N2-domain starts at nt 19 and ends at nt 411.

Figure 5.3: Cloning site of pComb3H5BHis with important restriction sites. The following abbreviations were used: P, lac-promotor; V_K, variable kappa light chain domain; V_H, variable heavy chain domain; ompA, procaryotic leader sequence; N2 is linked to V_H by a Gly_4Ser_1-linker; V_H is linked to V_K by a $(Gly_4Ser_1)_3$-linker.

Figure 6.1: Scheme of the pComb3H5BHis-plasmid and the fully expressed M13-phage. At the top the organization of leader (L) ompA, V_H, V_K and gene III is shown. A representative expressed M13-phage-particle (bottom) displays on its surface the phenotype of a certain scFv-fragment consisting of V_H and V_K linked with its C-terminus to the gene III product and with its N-terminus in the N2-domain and contains the corresponding genotype as single-stranded DNA encoding said protein elements as a single polypeptide chain.

Figure 6.2: ELISA-analysis of 17-1A-specific scFv protein fragments generated by the method of invention. Periplasma preparations of soluble scFv protein fragments containing the N2-domain at their N-terminus and consisting of one single mouse Vkappa- and one single Vheavy chain-domain, respectively were added pure to an ELISA-plate that had been coated with soluble 17-1A antigen. Detection was performed by a murine IgG1 anti-his-tag antibody followed by a peroxidase conjugated polyclonal goat anti mouse-Ig(Fc) antibody. The ELISA was developed by an ABTS-substrate solution as described in Example 8. The OD-values (y-axis) were measured at 405 nm by an ELISA-reader. Clones are presented on the x-

axis, the lower number indicates the round of panning, the number above indicates the tested clone of this round. Clones 0-1 to 0-9 have a combination of unselected scFv-fragments and therefore can be seen as negative controls, the positive control is an anti 17-1A / anti-CD3 bispecific single chain Fv antibody (Mack, Proc. Natl. Acad. Sci. USA 92 (1995) 7021-7025).

Figure 6.3: DNA- and protein-sequence of the mouse scFv fragment 3-1. Numbers indicate the nucleotide (nt) positions, amino acids (aa) are presented in single letter code. The first four aa of the V_H-fragment are encoded by the plasmid pComb3H5BHis. The encoding DNA sequence for the V-region of the heavy chain starts at nt 1 and ends at nt 360 followed by a $(G_4S_1)_3$-linker. The coding DNA sequence for the V-region of the kappa chain starts at nt 406 and ends at nt 726.

Figure 6.4: DNA- and protein-sequence of the mouse scFv fragment 3-5. Numbers indicate the nucleotide (nt) positions, amino acids (aa) are presented in single letter code. The first four aa of the V_H-fragment are encoded by the plasmid pComb3H5BHis. The encoding DNA sequence for the V-region of the heavy chain starts at nt 1 and ends at nt 372 followed by a $(G_4S_1)_3$-linker. The coding DNA sequence for the V-region of the kappa chain starts at nt 418 and ends at nt 753.

Figure 6.5: DNA- and protein-sequence of the mouse scFv fragment 3-8. Numbers indicate the nucleotide (nt) positions, amino acids (aa) are presented in single letter code. The first four aa of the V_H-fragment are encoded by the plasmid pComb3H5BHis. The encoding DNA sequence for the V-region of the heavy chain starts at nt 1 and ends at nt 360 followed by a $(G_4S_1)_3$-linker. The coding DNA sequence for the V-region of the kappa chain starts at nt 406 and ends at nt 726.

Figure 6.6: DNA- and protein-sequence of the mouse scFv fragment 4-1. Numbers indicate the nucleotide (nt) positions, amino acids (aa) are

presented in single letter code. The first four aa of the V_H-fragment are encoded by the plasmid pComb3H5BHis. The encoding DNA sequence for the V-region of the heavy chain starts at nt 1 and ends at nt 360 followed by a $(G_4S_1)_3$-linker. The coding DNA sequence for the V-region of the kappa chain starts at nt 406 and ends at nt 744.

Figure 6.7: DNA- and protein-sequence of the mouse scFv fragment 4-4. Numbers indicate the nucleotide (nt) positions, amino acids (aa) are presented in single letter code. The first four aa of the V_H-fragment are encoded by the plasmid pComb3H5BHis. The encoding DNA sequence for the V-region of the heavy chain starts at nt 1 and ends at nt 360 followed by a $(G_4S_1)_3$-linker. The coding DNA sequence for the V-region of the kappa chain starts at nt 406 and ends at nt 726.

Figure 6.8: DNA- and protein-sequence of the mouse scFv fragment 4-7. Numbers indicate the nucleotide (nt) positions, amino acids (aa) are presented in single letter code. The first four aa of the V_H-fragment are encoded by the plasmid pComb3H5BHis. The encoding DNA sequence for the V-region of the heavy chain starts at nt 1 and ends at nt 372 followed by a $(G_4S_1)_3$-linker. The coding DNA sequence for the V-region of the kappa chain starts at nt 417and ends at nt 753.

Figure 6.9: DNA- and protein-sequence of the mouse scFv fragment 5-3. Numbers indicate the nucleotide (nt) positions, amino acids (aa) are presented in single letter code. The first four aa of the V_H-fragment are encoded by the plasmid pComb3H5BHis. The encoding DNA sequence for the V-region of the heavy chain starts at nt 1 and ends at nt 348 followed by a $(G_4S_1)_3$-linker. The coding DNA sequence for the V-region of the kappa chain starts at nt 394 and ends at nt 717.

Figure 6.10: DNA- and protein-sequence of the mouse scFv fragment 5-10. Numbers indicate the nucleotide (nt) positions, amino acids (aa) are

presented in single letter code. The first four aa of the V_H-fragment are encoded by the plasmid pComb3H5BHis. The encoding DNA sequence for the V-region of the heavy chain starts at nt 1 and ends at nt 360 followed by a $(G_4S_1)_3$-linker. The coding DNA sequence for the V-region of the kappa chain starts at nt 406 and ends at nt 744.

Figure 7: DNA- and protein-sequence of the mouse scFv fragment 5-13. Numbers indicate the nucleotide (nt) positions, amino acids (aa) are presented in single letter code. The first four aa of the V_H-fragment are encoded by the plasmid pComb3H5BHis. The encoding DNA sequence for the V-region of the heavy chain starts at nt 1 and ends at nt 360 followed by a $(G_4S_1)_3$-linker. The coding DNA sequence for the V-region of the kappa chain starts at nt 406 and ends at nt 744.

Figure 8.1: ELISA analysis of nine cell-culture supernatants (primary selection step (PS)) obtained from CHO cells transfected with the expression plasmids pEF-DHFR + CTI + CD80 + scFv 17-1A clones 3-1 to 5-13. 96 well U bottom ELISA plates were incubated with 50 µl of soluble 17-1A antigen (50µg/ml) per well. Antibody constructs as culture supernatants were added pure and at following dilutions: 1:2, 1:4, 1:8. Detection was performed by a CD80-specific monoclonal antibody diluted 1:1000 in PBS 1%BSA followed by a polyclonal peroxidase-conjugated Goat Anti-Mouse IgG-antibody (Fc-specific) (dianova, Hamburg) diluted 1:5000. The ELISA was finally developed by adding the ABTS substrate solution as described in Example 8. For negative controls, the plates were incubated with PBS instead of bifunctional antibody constructs. The OD-values were measured by an ELISA reader at 405 nm.

Figure 8.2: ELISA analysis of nine cell-culture supernatants (1. Amplification step (20 nM MTX) (1. Amp)) obtained from CHO cells transfected with the expression plasmids pEF-DHFR + CTI + CD80 + scFV 17-1A clones 3-1 to 5-13. 96 well U bottom ELISA plates were incubated with 50 µl of soluble 17-1A antigen (50µg/ml) per well. Antibody constructs as culture supernatants were

added pure and at following dilutions: 1:2, 1:4, 1:8. Detection was performed by a CD80-specific monoclonal antibody diluted 1:1000 in PBS 1% BSA followed by a polyclonal peroxidase-conjugated Goat Anti-Mouse IgG-antibody (Fc-specific) (dianova, Hamburg) diluted 1:5000. The ELISA was finally developed by adding the ABTS substrate solution as described in Example 8. For negative controls, the plates were incubated with PBS instead of bifunctional antibody constructs. The OD-values were measured by an ELISA reader at 405 nm.

Figure 8.3: ELISA analysis of two cell-culture supernatants (primary selection step (PS)) from 17-1A specific bifunctional CD80-scFv-constructs, which were generated as described in Example 3 and 4. 96 well U bottom ELISA plates were incubated with 50 µl of soluble 17-1A antigen (50µg/ml) per well. Antibody constructs as culture supernatants were added pure and at following dilutions: 1:2, 1:4, 1:8. Detection was performed by a CD80-specific monoclonal antibody diluted 1:1000 in PBS 1% BSA followed by a polyclonal peroxidase-conjugated Goat Anti-Mouse IgG-antibody (Fc-specific) (dianova, Hamburg) diluted 1:5000. The ELISA was finally developed by adding the ABTS substrate as described in Example 8. For negative controls, the plates were incubated with PBS instead of bifunctional antibody constructs. As positive control served a supernatant generated in Example 7. The OD-values were measured at 405 nm using an ELISA-reader.

Figure 8.4: ELISA analysis of two cell-culture supernatants (1. Amplification step (20 nM MTX) (1. Amp)) from 17-1A specific bifunctional CD80-scFv-constructs, which were generated as described in Example 3 and 4. 96 well U bottom ELISA plates were incubated with 50 µl of soluble 17-1A antigen (50µg/ml) per well. Antibody constructs as culture supernatants were added pure and at following dilutions: 1:2, 1:4, 1:8. Detection was performed by a CD80-specific monoclonal antibody diluted 1:1000 in PBS 1%BSA followed by a polyclonal peroxidase-conjugated Goat Anti-Mouse IgG-antibody (Fc-specific) (dianova, Hamburg) diluted 1:5000. The ELISA was finally

developed by adding the ABTS substrate solution as described in Example 8. For negative controls, the plates were incubated with PBS instead of bifunctional antibody constructs. As positive control a supernatant generated in Example 7 was used. The OD-values were measured at 405 nm using an ELISA-reader.

Figure 9.1: Binding studies of 17-1A specific bifunctional CD80-scFv-constructs on 17-1A transfected (filled lines) and untransfected CHO cells (broken lines) detected by flow cytometry. $5x10^5$ cells were incubated in 50 µl undiluted cell-culture supernatant containing the corresponding bifunctional construct. Bound bifunctional CD80-scFv-constructs were detected by a monoclonal anti-CD80 antibody (Immunotech. Cat. No.: 1449) diluted 1:20 in 50 µl PBS. Incubation conditions were the same as described in Figure 8.5. Bound CD80-antibody was finally detected by a fluorescein conjugated polyclonal Goat Anti-Mouse IgG + IgM (H+L) antibody diluted 1:100 in PBS. Incubation was again carried out for 30 minutes on ice. For the fixation of fluorescein-labeled cells 1% paraformaldehyd in PBS was used. As first negative control untransfected CHO was used. The second negative control contained 17-1A-transfected cells that were incubated with PBS instead of bifunctional CD80-scFv-constructs. Cells were analysed by flow cytometry on a FACS scan (Becton Dickenson).

Figure 9.2: FACS-Control of the CHO cells after transfection with 17-1A.
The expression of transmembrane 17-1A was increased by stepwise gene amplification induced by subsequent addition of increasing concentrations of the DHFR inhibitor MTX to a final concentration of 500nM, with the concentration steps in between 20nM and 100nM. These cells were tested for membrane expression of 17-1A by flow cytometry at a concentration of 10µg/ml of the 17-1A-specific antibody M79 (Göttlinger, Int. J. Cancer 38 (1986), 47-53) followed by a FITC-labeled polyclonal Goat Anti Mouse IgG + IgM (H+L) antibody diluted 1:100 in PBS. As negative control untransfected

CHO cells were used whereas the 17-1A-positive human gastric cancer cell-line Kato, obtained from ATCC served as positive control.

Figure 10: Principle of constructing bifunctional single-chain proteins

Figure 11: Structural comparison between wildtype phage, conventional phage display and phage display according to the method of the invention.

Tab. 1: Primer sets for the amplification of the V_H- and VK-DNA-fragments (5' to 3')

The following Examples illustrate the invention:

Example 1: CD80-M79scFv constructs

1.1 CD80 - M79 scFv (V_L/V_H) construct with short $(Gly_4Ser_1)_1$ linker

A protein was constructed that consists of the single-chain Fv fragment (scFv) of the murine anti 17-1A antibody M79 and the extracellular part of the human costimulatory protein CD80 (B7-1) connected by a $(Gly_4Ser_1)_1$ linker (Figure 1.1). The M79 antibody was obtained as described by Göttlinger (1986) Int.J.Cancer:38, 47-53. The M79 scFv fragment was cloned as described by Mack. Proc. Natl. Acad. Sci. U.S.A.. 92 (1995) 7021-7025. The complete plasmid was cloned in several steps. First a poly-linker designated CTI was inserted into the Bluescript KS vector (GenBank® accession number X52327) using the restriction enzyme cleavage sites XbaI and SalI (Boehringer Mannheim). The introduction of the polylinker CTI provided additional cleavage sites as well as the sequence encoding the $(Gly_4Ser_1)_1$ linker a six-amino acid histidine tag and a stop codon as shown in Figure 1.2..The vector Bluescript KS + CTI was prepared by cleavage with the restriction enzymes EcoRV and XmaI (Boehringer Mannheim and New England Biolabs) in order to ligate it (T4 DNA, Ligase Boehringer Mannheim) with the M79 scFv

fragment cleaved by EcoRV and BspEI (New England Biolabs). The resulting vector Bluescript KS+CTI+M79 scFv again was cleaved with EcoRI (Boehringer Mannheim) and BspEI in order to insert the CD80 DNA-fragment which was previously prepared using the same enzymes. Prior to subcloning, the CD80 fragment was obtained by polymerase chain reaction (PCR) using specific oligonucleotide primers complementary to the 5′ and 3′ ends of the nucleotide sequence encoding the extracellular part of CD80 (Freeman G.J et.al. J.Immunol.143,(1989) 2714 - 2722.). These primers also introduced an EcoRI and a BspEI cleavage site (5′CD80 Primer: 5′GCA GAA TTC ACC ATG GGC CAC ACA CGG AGG CAG 3′; 3′CD80 Primer: 5′TGG TCC GGA GTT ATC AGG AAA ATG CTC TTG CTT G 3′) The cDNA template used for this PCR was prepared by reverse transcription of the total RNA prepared from the Burkitt-lymphoma cell line Raji according to standard procedures (Sambrook, Molecular Cloning; A Laboratory Manual, 2nd Edition, Cold Spring Harbour Laboratory Press, cold Spring Habour, New York (1989)).

The CD80 costimulatory protein belongs to the Ig superfamily. It is a heavily glycosylated protein of 262 amino acids. A more detailed description was published by Freeman G.J et.al. J.Immunol.143,(1989) 2714 - 2722.

In the last step, the whole CD80-M79scFv (V_L/V_H) DNA fragment (Figure 1.3.1.) was isolated by cleaving the vector Bluescript KS+CTI+CD80-M79scFv (V_L/V_H) with EcoRI and SalI (Boehringer Mannheim) and subsequently introduced into the eukaryontic expression vector pEF-DHFR described in Mack et.al. Proc. Natl. Sci. U.S.A. 92 (1995) 7021-7025 containing the dihydrofolatereductase gene as selection marker. The final plasmid was linearized with the restriction enzyme NdeI (Boehringer Mannheim) and transfected into CHO cells by electroporation. The electroporation conditions were 260V/960µFD using a BioRad Gene Pulser™. Stable expression was performed in DHFR deficient CHO-cells as described by Kaufmann R.J. (1990) Methods Enzymol. 185, 537-566. The cells were grown for selection in nucleoside free α-MEM medium supplemented with

10% dialysed FCS and 2 mM L-glutamine. For production of the bifunctional CD80-M79 scFv (V_L/V_H) construct, cells were grown in rollerbottles (Falcon) for 7 days in 300ml culture medium. The protein was purified via its His-tag attached to the C-terminus (see Figure 1.1.) by using a Ni-NTA-column (Mack et.al., Proc. Natl. Acad. Sci. U.S.A. 92 (1995)7021-7025). To analyse the binding properties different ELISA assay were performed:

1.1.1 ELISA with cell culture supernatant using anti-His-tag detection

Binding to the 17-1A-antigen was analysed using soluble 17-1A-antigen obtained as described (Mack, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 7021-7025) by stable expression in CHO-cells of the DNA encoding the first 264 amino acids of the 17-1A antigen also known as GA 733-2 (Szala, Proc. Natl. Acad. Sci. U.S.A. 87 (1990) 3542-3546) followed by a stop codon.. The antigen was immobilized on 96 well U bottom ELISA plates (nunc maxisorb) at a concentration of 50µg/ml phosphat buffered saline PBS. Coating was carried out at 4°C for 12 hours with 50µl followed by washing once with (PBS) 0,05%Tween. The ELISA was then blocked for 1 hour with PBS/3%bovine serum albumin (BSA) and washed again once. Now the cell-culture supernatant was added undiluted and at several dilutions and incubated for 2 hours. As detection system a murine IgG1 anti His-tag antibody (dianova, Hamburg) diluted 1:200 and a peroxidase conjugated polyclonal goat anti mouse IgG (Fc) (dianova, Hamburg) antibody were applied sequentially. The ELISA was developed by adding ABTS-substrate solution (2´2 Azino-bis (3-Ethlbenzthiazoline-6-Sulfonic Acid), SIGMA A-1888, Steinheim) as described in Example 8. The result was measured by an ELISA-Reader at OD 405 nm; results are shown in Figure 1.4. Obviously no binding activity could be measured. As negative controls, the plates were incubated with PBS instead of antibody constructs. As positive control served the anti-17-1A/anti-CD3 bispecific-single-chain antibody described previously (Mack, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 7021-7025).

1.1.2 ELISA with cell culture supernatant using anti-CD80 detection

Immobilization of 17-1A-antigen, blocking and the incubation of cell culture supernatants was performed as described above. Detection was carried out with a murine IgG1 anti-CD80-antibody diluted 1:1000 (dianova, Hamburg) followed by a peroxidase conjugated polyclonal goat anti-mouse IgG (Fc)-antibody diluted 1:5000 (dianova, Hamburg). The ELISA was developed with ABTS-substrat solution and OD-values were measured as described above, however, again no 17-1A-binding activity could be detected. As positive control, the anti-17-1A/anti-CD3 bispecific-single-chain antibody (Mack, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 7021-7025) was used and detected with the described anti-His-tag antibody. Results are shown in Figure 1.5

1.1.3 ELISA-analysis of purified recombinant CD 80-M79scFv-construct

As the ELISAs with cell-culture supernatants detecting specific antigen binding were all negative, soluble CD80-M79scFv was obtained by protein purification from supernatant of a roller bottle culture (300ml) in order to exclude the possibility that no recombinant protein was secreted into the supernantant. The purification was carried out using a Nickel-NTA-column as described (Mack, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 7021-7025). ELISA wells were coated with the protein eluted from the Nickel-NTA-column. Detection of the bifunctional CD80-M79scFv-construct was performed independently of its 17-1A-antigen binding activity by using either an anti His-tag antibody (see Example 1.1.1.) as well as an anti-CD80 antibody (see Example 1.1.2.) in separate experiments followed by an anti-mouse IgG(Fc) antibody, respectively. Development of the ELISA as well as the measurement of the OD-values was carried out as described above. The results are shown in Figure 1.6., confirming the presence of the CD80-M79scFv-construct in the cell culture supernatant.

1.2 CD80 - M79 scFv (V_H/V_L) construct with $(Gly_4Ser_1)_1$ linker

To change the arrangement of the Ig variable regions within the M79scFv fragment from V_L/V_H to V_H/V_L a two step fusion PCR using oligonucleotide primers 5´V_HB5RRV:AGG TGT ACA CTC CGA TAT C(A,C)A (A,G)CT GCA G(G,C)A GTC (A;T)GG, 3´V_HGS15, 5´V_LGS15 ,3´V_LBspE1 (for sequences of the three last oligonucleotides see Example 2.1) was performed according to the procedure described by Mack, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 7021-7025 (see also Example 2.1.) The PCR-fragment encoding the V_H/V_L-scFv-fragment was cleaved with the restriction enzymes EcoRV/BspEI and inserted into the vector Bluescript KS + CTI already prepared by cleavage with EcoRV/XmaI (see Example 1.1.). Next, the inverted M79scFv (V_H/V_L) fragment was excised with the restriction enzymes BspEI/SalI and introduced into the plasmid pEF-DHFR+CTI + CD80-M79scFv (V_L/V_H) using BspEI/SalI thus replacing the M79scFv- V_L/V_H fragment (see Figure 1.3.2.). Transfection and cell culture procedures were carried out as described above. Analysis of antigen binding was performed using the described 17-1A-ELISA (Example 1.1.2.). However, no 17-1A binding activity of the alternatively arranged CD80-M79scFv-construct could be detected. Results are shown in Figure 1.7.

1.3 CD80 - M79 scFv (V_H/V_L) construct with a long $(Gly_4Ser_1)_3$ linker

First, the M79scFv (V_H/V_L) fragment was obtained by a two step fusion PCR as described in Example 1.2. The PCR fragment encoding the V_H/V_L-scFv-fragment was cleaved with the restriction enzymes EcoRV/BspEI and subcloned into the Bluescript KS +CTI vector cleaved EcoRV/XmaI (see Example 1.1). In a further step a longer Glyin-Serin linker $(Gly_4Ser_1)_3$ consisting of 15 amino acids was introduced. Therefore, another oligonucleotide linker (ACCGS15BAM), which was designed to encode the $(Gly_4Ser_1)_3$ linker and to provide BspEI and BamHI compatible overhangs had to be inserted into the Bluescript KS + CTI + M79 scFv (V_H/V_L) (Example 1.2). The nucleotide sequence of the linker is shown in Figure 1.8.

The plasmid Bluescript KS + CTI + M79 scFv (V_H/V_L) including the coding sequence of the $(Gly_4Ser_3)_3$ linker was cleaved with BspEI and SalI and the resulting DNA-fragment $(Gly_4Ser_1)_3$+M79scFv (V_H/V_L) was inserted into the BspEI/SalI-cleaved vector pEF-DHFR that contains the CD80-coding fragment (Example 1.1) thus replacing the M79scFv (V_L/V_H) fragment together with the short $(Gly_4Ser_1)_1$ linker (see Figure 1.3.3). For transfection and cell culture procedure see Example 1.1. Antigen specific binding was analysed by 17-1A ELISA as described above (Example 1.1.1) and detection of functional recombinant protein in the cell-culture supernatant was performed with an anti His-tag antibody followed by an anti mouse IgG (Fc) antibody (compare Example 1.1.1). The anti-17-1A/anti-CD3 bispecific-single-chain antibody (Mack, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 7021-7025) served as positive control. Development of the ELISA and measurement of the OD values was carried out as described above (Example 1.1.1). However, no antigen binding was detectable. Results are shown in Figure 1.9.

Example 2: CD80 - M74 scFv construct with either short $(Gly_4Ser_1)_1$ or long $(Gly_4Ser_1)_3$ linker as well as (V_H/V_L) or (V_L/V_H)-domain arrangement

A protein was constructed that consists of the single-chain Fv fragment (scFv) of the anti 17-1A antibody M74 and the costimulatory protein CD80 connected by a (Gly_4Ser_1) linker (Figure1.1). The M74 antibody was obtained as described by Göttlinger (1986) Int. J. Cancer: 38, 47-53. V_L and V_H of M74 were cloned from the total RNA of the corresponding hybridoma cell line as described by Orlandi (1989) Proc. Natl. Acad. Sci. USA 86, 3833-3837 and sequenced. The plasmids containing V_L and V_H of the M74 antibody respectively were used as templates for a two-step fusions-PCR resulting in M74 scFv-fragments with either the domain arrangement V_L/V_H or the alternative arrangement V_H/V_L. Regarding the V_L/V_H arrangement, the primers for M74 V_L were 5´V_LB5RRV (5´AGG TGT ACA CTC CGA TAT CCA GCT

GAC CCA GTC TCC A3′) and 3′V_LGS15 (5′GGA GCC GCC GCC GCC AGA ACC ACC ACC ACC TTT GAT CTC GAG CTT GGT CCC3′), for M74 V_H 5′M74V_HGS15 (5′GGC GGC GGC GGC TCC GGT GGT GGT GGT TCT CAG GT(GC) (AC)A(AG) CTG CAG (GC)AG TC(AT) GGA CCT GAG CTG GTG AAG CCT GGG GCT TCA GTG AAG ATT TCC TGC 3′) and 3′V_HBspEI (5′AAT CCG GAG GAG ACG GTG ACC GTG GTC CCT TGG CCC CAG3′). Regarding the V_H/V_L-arrangement the primers for M74 V_H were 5′M74V_HEcoRV (5′TCC GAT ATC (AC)A(AG) CTG CAG (GC)AG TC(AT) GGA CCT GAG CTG GTG AAG CCT GGG GCT TCA GTG AAG ATT TCC TGC 3′) and 3′V_HGS15 (5′GGA GCC GCC GCC GCC AGA ACC ACC ACC ACC TGA GGA GAC GGT GAC CGT GGT CCC TTG GCC CCA G 3′), for M74 V_L 5′V_LGS15 (5′GGC GGC GGC GGC TCC GGT GGT GGT GGT TCT GAC ATT CAG CTG ACC CAG TCT CCA3′) and 3′V_LBspEI (5′AAT CCG GAT TTG ATC TCG AGC TTG GTC CC3′). In the first PCR step the corresponding V_H-and-V_L fragments were obtained using the following PCR-program: denaturation at 94 °C for 5 min., annealing at 37°C for 2 min., elongation at 72°C for 1 min. for the first cycle; denaturation at 94°C for 1 min., annealing at 37°C for 2 min., elongation at 72°C for 1 min. for 6 cycles; denaturation at 94°C for 1 min., annealing at 55°C for 1 min., elongation at 72°C for 45 sec and 18 cycles ; terminal extension at 72°C for 2 min.). The purified PCR-fragments of V_H and V_L were then used for the second step of the fusion PCR using the following primers for M74 scFv V_L/V_H: 5′V_LB5RRV and 3′V_H BspEI, as well as 5′M74V_HEcoRV and 3′V_LBspEI for M74 scFV V_H/V_L The following PCR-program was used: denaturation at 94°C for 5 min. once; denaturation at 94°C for 1 min., annealing at 55°C for 1 min., elongation at 72°C for 1:30 min. and 8 cycles; terminal extension at 72°C for 2 min.). The next step was to clone both M74 scFv sequences into the plasmid Bluescript KS+CTI (see Example 1) by cleaving the fragments with EcoRV/BspEI and the vector with EcoRV/XmaI. To obtain constructs with different linker length, the following strategy was used :

For generating the CD80-M74scFv-construct with the V_H/V_L-and the V_L/V_H-arrangement respectively and a short $(Gly_4Ser_1)_1$ linker, the M74 scFv fragment (V_H/V_L) as well as the M74 scFv fragment (V_L/V_H) were excised from Bluescript KS+CTI respectively and each inserted into the vector pEF-DHFR+CTI+ CD80-M79scFv(V_L/V_H) (see Example 1.1) using the restriction enzymes BspEI and SalI (see Figure 1.3.4 and 1.3.5). For the transfection in CHO-cells and the cell-culture conditions see Example 1.1.

For generating the CD80-M74 scFv-construct with the V_H/V_L-and the V_L/V_H-arrangement respectively, each containing a long $(Gly_4Ser_1)_3$ linker, the M74 scFv fragments were excised from the vector Bluescript KS+CTI as described above and introduced into the plasmid Bluescript KS + CTI + M79scFv (V_H/V_L) including the long linker (see Example 1.3) by cleaving vector and fragments with EcoRV and SalI respectively thereby replacing the M79 specificity with M74 (V_H/V_L) or M74 (V_L/V_H). The last step prior to transfection was to introduce M74 (V_H/V_L) or M74 (V_L/V_H) into the pEF-DHFR + CTI + CD80-M79scFv (V_H/V_L) vector respectively using the restriction enzymes BspEI and SalI (see Figure 1.3.7 and 1.3.8) thus resulting in plasmids with all the requirements for the expression in CHO-cells of CD80-M74 scFv-constructs either with the V_H/V_L-or the V_L/V_H-domain arrangement and a long $(Gly_4Ser_1)_3$ linker, respectively. For the transfection in CHO-cells and the cell-culture conditions see Example 1.1. The four different constructs (CD80 -$(Gly_4Ser_1)_1$ - M74 (V_H/V_L), CD80 -$(Gly_4Ser_1)_1$ - M74 (V_L/V_H), CD80 -$(Gly_4Ser_1)_3$ - M74 (V_H/V_L), CD80 -$(Gly_4Ser_1)_3$ - M74 (V_L/V_H)) were all tested for binding to the 17-1A-antigen using cell-culture supernatants as well as purified from culture supernatant using Nickel-NTA-columns as described in Example 1.1.1 and 1.1.3 respectively. The ELISA was performed as described and detection was carried out by using either an anti His-tag antibody or an anti CD80 antibody followed by a peroxidase conjugated anti-mouse-IgG (Fc) antibody (see Example 1.1.1) respectively. Despite the fact that recombinant protein could be purified from all four supernatants (data not shown), no binding to the 17-1A-antigen could be detected as shown in Figure 2.1 and 2.2.

Example 3: CD80 - VD4.5VK8 scFv(V_H/V_L) construct with short $(Gly_4Ser_1)_1$ linker

In a further Example a human anti-17-1A antibody (VD4.5VK8) selected in vitro by the phage display method from a combinatorial antibody library was chosen to analyse its antigen-binding activity at the C-Terminus of a bifunctional single-chain construct as in Examples 1, 2 and 4 and as illustrated in Figure1.1. The V_H -and V_L-chain of VD4.5VK8 were available in the form of cloned DNA fragments with known nucleotide sequence (Figure 3.1 and 3.2) and served as template molecules for PCR using the following primers: for V_H: 5'V_H1357 5'-AGG TGC AGC TGC TCG AGT CTG G-3, and 3'huV$_H$BstEII 5'-CTG AGG AGA CGG TGA CC'-3; for V_L: 5'VK3 GAG CCG CAC GAG CCC GAG CTC GTG (AT)TG AC(AG) CAG TCT CC-3', and 3'huVkBsiWI/SpeI 5'-GAA GAC ACT AGT TGC AGC CAC CGT ACG TTT (AG)AT-3'). The V_H-respectively V_L-chains were introduced into a newly constructed vector designated pComb3H5BHis and described in Example 5. VD4.5VK8 V_H was subcloned with XhoI and Bst EII, VD4.5VK8 V_L with SacI and SpeI resulting in the plasmid: pCOMB3H5BHis+VD4.5VK8 V_H+V_L. By using the pComb3H5BHis-vector a fusion PCR was no longer necessary to obtain a scFv-antibody fragment with the domain arrangement V_H/V_L.

To analyse the 17-1A-binding activity of the VD4.5VK8 scFv-fragment the N2 fragment (see Example 5) was excised by the restriction enzymes XhoI and SalI. The compatible vector ends were religated; the ligation product was transformed into E.coli XI 1 Blue and periplasmatic protein expression was induced by adding IPTG. Periplasma preparation was carried out and the resulting sample was directly used for the ELISA-based analysis of 17-1A antigen binding activity as described in Example 5. The wells were coated with soluble 17-1A and bound scFv fragments were detected with a murine anti His-tag antibody diluted 1:200 followed by an anti-mouse IgG (Fc) antibody (see Example 1.1.1) diluted 1:5000. Development of the ELISA and measurement of the OD-values was performed as described in Example

1.1.1. As positive control anti 17-1A antibody clone 3-5 obtained by the method of the invention was used (see Example 6). The results are shown in Figure 3.3 and reveal significant binding of the free monovalent VD4.5.VK8 scFv-fragment to immobilized 17-1A antigen. The next step in generating the bifunctional CD80-VD4.5VK8-scFv-construct was to cleave the plasmid designated Bluescript KS + CTI+L-F-NS3 Frame, deleted of the Bluescript-derived NotI-site and containing an extended polylinker (for the sequence see Figure 3.4), by the enzymes EcoRI and NotI to subclone the EcoRI/NotI VD4.5VK8 fragment from vector pCOMB3H5BHis+VD4.5VK8 V_H+V_L described above.

As the last step in generating the bifunctional CD80-VD4.5VK8-scFv-construct, the VD4.5VK8- scFv-fragment was excised from the vector Bluescript KS+CTI+L+F+NS3 Frame using the restriction enzymes BspEI and SalI and subcloned into the plasmid pEF-DHFR+CTI+CD80-M79scFv (V_L/V_H) (see Examples 1.1 and 1.2) cleaved with the same enzymes and thereby replacing the M79 scFv fragment by that of the human antibody VD4.5VK8 (see Figure 1.3.8) Transfection into CHO-cells and cell-culture procedures were performed as described in Example 1.1.1. The 17-1A-antigen-binding activity was analysed by ELISA (Figures 8.3 and 8.4) and flowcytometry (Figure 9.1 and 9.2) described in detail in Examples 8 and 9; however, no binding to the 17-1A-antigen could be detected by either method.

Example 4: CD80- MACHscFv antibody construct

Another murine anti-17-1A-antibody (MACH) obtained by the method described by Göttlinger (1986) Int. J. Cancer:38, 47-53., was analysed with respect to the antigen binding activity of its scFv-fragment at the C-terminus of a bifunctional single-chain construct. The corresponding immunoglobulin variable regions V_L and V_H were cloned by RT-PCR according to Orlandi, (1989) Proc. Natl. Acad. Sci. USA: 86, 3833-3837 from the total RNA prepared from the hybridoma cell line and subsequently expressed in mammalian cells as chimeric antibody of the human $IgG1_{kappa}$ -Isotype

according to Orlandi (1989) Proc. Natl. Acad. Sci. U.S.A.: 86, 3833-3837. The recombinant antibody proved to bind to the 17-1A-antigen resembling its murine parent antibody as determined by 17-1A-ELISA using the culture supernatants of the transfected and the hybridoma cell line, respectively. Detection of bound antibody was performed with an anti-human-or an anti-murine immunoglobulin antibody, respectively. Development of the ELISA and measurement of OD-values was performed as described in Example 8. The results are shown in Figure 4.

The Vk and Vh domains were cloned into pComb3H5BHis (according to Examples 3 and 5). The murine anti-17-1A-scFv-fragment was introduced into plasmid the pEF-DHFR+CTI+ CD80-VD4.5VK8 (see Example 3) using the restriction enzymes BspEI and NotI, thus replacing the 17-1A-specific VD4.5VK8scFv fragment (Figure 1.3.9). The obtained expression plasmid was then transfected into CHO cells as described in Example 1.1. The 17-1A binding activity on was analysed by ELISA (Figures 8.3 and 8.4) and flowcytometry (Figure 9.1 and 9.2) described in detail in Examples 8 and 9; however, no binding to the 17-1A antigen could be detected by either method.

Example 5: Construction of the phagmid vector pComb3H5BHis

As a starting point for a phage display vector applicable for the in vitro selection of antibody fragments according to the method of the present invention the vector pComb3H, a derivative of pComb3 (Barbas, Proc. Natl. Acad. Sci. U.S.A. 88 (1991) 7978-7982) was used (for cloning sites see Figure 5.1), providing:

- the bla-gene enabling carbenicilline resistance selection for positive transformation and infection with recombinant phage particles
- a procaryotic leader sequences for protein excretion of functional antibody fragments into bacterial periplasma
- an inducible lac-promotor for high protein productivity

- the coat domain CT of the M13 phage gene III product necessary for anchoring antibody fragments on the surface of filamentous phage (phage display).

For the detection and isolation of proteins expressed in the periplasma of E.coli, especially small scFv fragments, a His tag is highly preferable. Therefore the first step was to subclone a DNA-sequence encoding six Histidine residues downstream of the gene III sequence.
The pComb3H vector was cleaved with NheI and a double stranded oligonucleotide with suitable ends was inserted by ligation. The double stranded oligomer encoding the six His residues was created through annealing of the two 5'-phosphorylated primers His6s and His6as (at 94°C, 10 min.; 65 °C, 30 min.; 52 °C 30 min. and 30 °C 10 min.).

His6s: 5'-CTAGCCATCACCATCACCATCACA-3´

His6as: 5'-CTAGTGTGATGGTGATGGTGATGG-3´

The primer ends were designed in a way that after fusion with the vector the 3' NheI restriction site was destroyed whereas the 5' NheI cleavage site remained intact.
The insert was sequenced to confirm successful cloning and the new vector designated pComb3HHis.

For the purpose of creating scFv-fragments linked to the gene III product with the C-terminus of the light chain variable domain (VK), a totally new multiple cloning site (mcs) had to be subcloned.

The first part of the original mcs of pComb3HHis was excised by SacI-XhoI digestion. The resulting vector fragment was ligated with a double stranded (ds) DNA fragment created by annealing of two 5'-phosphorylated primers (5BFors;5BForas) giving rise to 5' SacI and 3'XhoI compatible overhangs and

destroying the original 5' SacI cleavage site. The annealing of the two primers was carried out at 94°C, 10 min.; 65 °C, 30 min.; 52 °C 30 min. and 30 °C 10 min.

Primer sequences:

5BFors:

5'-
GCAGCTGGTCGACAAATCCGGAGGTGGTGGATCCGAGGTGCAGCTGC-
3'

5BForas:

5'-
TCGAGCAGCTGCACCTCGGATCCACCACCTCCGGATTTGTCGACCAGCT
GCAGCT-3'

The insert was sequenced to confirm successful cloning and the new vector designated pComb3HForHis. The original heavy chain cloning stuffer was then excised with XhoI and SpeI, and the resulting vector fragment was ligated with another ds DNA-fragment, again created by annealing of two 5' phosphorylated primers (5BBacks; 5BBackas) under the same conditions used for the annealing of 5BFors and 5BForas.

Primer sequences:

5BBacks:

5'-
TCGAGCCCGGTCACCGTCTCCTCAGGTGGTGGTGGTTCTGGCGGCGGC
GGCTCCGGTGGTGGTGGTTCTGAGCTCGGGA-3'

5BBackas:

5'-
CTAGTCCCGAGCTCAGAACCACCACCACCGGAGCCGCCGCCGCCAGAA
CCACCACCACCTGAGGAGACGGTGACCGGGC-3'

The whole insert was again sequenced to confirm successful cloning and the new vector designated pComb3HmcsHis (Figure 5.2).

This vector provides all necessary cloning sites for the cloning of scFv antibody fragments, a procaryotic leader sequence for the transport of the recombinant proteins into the periplasma of E.coli, a linkage of scFV-fragments to the CT-domain of the geneIII-product of filamentous phage and after removal of the CT-encoding sequence a linkage to a histidine tag.
The last and most important step was to introduce a protein reducing the antigen binding activity of position-sensitive antibody fragments and being neutral to insensitive scFv-fragments so that its C-terminus will be fused to the N-terminus of subsequently cloned scFv-antibody-fragments.
The M13 gene III domain N2 corresponding to the amino acids 87 to 217 of the geneIII-product of bacteriophage fd (Beck, Nucl. Acid. Res. 5 (1978), 4495-4503) was chosen as a suitable protein to be fused to the N-terminus of scFv-fragments; unlike the complete geneIII-product, the N2-domain does not mediate phage infectivity.
The approximately 400bp N2-fragment was amplified by PCR (polymerase chain reaction) from VCSM13-phage (available from Stratagene) infected E.coli XL1blue (94°C, 4 min.; (94°C, 0,5 min.; 52°C, 1 min.; 72°C, 0,5 min.) x 40 cycles; 72°C, 10 min.; 30°C, 1 sec.) using the primers 5' N2 SalI and 3'N2 BspEI.

Primer sequences:

5' N2 SalI : 5'-GGTGTCGACACTAAACCTCCTGAGTACGG-3'

3'N2 BspEI : 5'-GCCTCCGGAAGCATTGACAGGAGGTTGAGG-3'

This fragment was then subcloned into the pComb3HmcsHis vector using the restriction sites, SalI and BspEI.
Correct subcloning was confirmed by DNA-sequencing. The resulting vector was designated pComb3H5BHis.
The sequence of its multiple cloning site is shown in Figure 5.2.
Figure 5.3 shows a plasmid map of pComb3H5BHis with a cloned scFv-antibody-fragment.
Unless otherwise stated, the procedures used followed that described in Sambrook, Molecular Cloning, 'A Laboratory Manual', 2nd Edition, Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY (1989).

Example 6: Construction of the combinatorial antibody library and phage display

For immunization 25 µg soluble 17-1A-antigen in 100µl PBS were mixed with 100 µl incomplete Freuds Adjuvance (IFA) and injected subcutaneously into one mouse. After two and five weeks injection was repeated with the same amount of antigen mixed with the same volume (100 µl) of IFA, respectively.
Four weeks after the first injection, successful immunization was analysed by the 17-1A ELISA (see Example 8) using mouse-serum diluted 1:5, 1:50, and 1:500 followed by a peroxidase conjugated anti-mouse Ig-antibody. A strong signal was obtained in all concentrations compared to negative and cross-reactivity controls.

Three days after the third injection the murine spleen cells were harvested for the preparation of total RNA according to Chomczynski, Analytical biochemistry 162 (1987) 156-159.

A library of murine immunoglobuline (Ig) light chain (kappa) variable region (VK) and Ig heavy chain variable region (V_H) DNA-fragments was constructed by RT-PCR on murine spleen RNA using VK- and V_H specific primer. cDNA was synthesized according to standard protocols (Sambrook, Cold Spring Harbour Laboratory Press 1989, second edition).

The primer sets (Table 1) were chosen to give rise to a 5'- XhoI and a 3'-BstEII recognition site for the heavy chain V-fragments and to a 5'-SacI and a 3'- SpeI recognition site for VK.

For the PCR-amplification of the V_H DNA-fragments eight different 5'-V_H-family specific primers were each combined with one 3'-V_H primer; for the PCR-amplification of the VK-chain fragments seven different 5'-VK-family specific primers were each combined with one 3'-VK primer. Primer sets for the amplification of the V_H- and VK-DNA-fragments (5' to 3') are shown in Table 1.

The following PCR program was used for amplification: denaturation at 94 °C for 20 sec.; primer annealing at 52 °C for 50 sec. and primer extension at 72 °C for 60 sec. and 40 cycles, followed by a 10 min. final extension at 72 °C.

450 ng of the kappa light chain fragments (SacI-SpeI digested) were ligated with 1400 ng of the phagmid pComb3H5BHis (SacI-SpeI digested; large fragment). The resulting combinatorial antibody library was then transformed into 300 µl of electrocompetent Escherichia coli XL1 Blue cells by electroporation (2.5 kV, 0.2 cm gap cuvette, 25 µ FD, 200 Ohm, Biorad gene-pulser) resulting in a library size of 6×10^8 independent clones. After one hour of phenotype expression, positive transformants were selected for carbenicilline resistance encoded by the pComb3H5BHis vector in 100 ml of liquid super broth (SB)-culture over night.

Cells were then harvested by centrifugation and plasmid preparation was carried out using a commercially available plasmid preparation kit (Qiagen).
2800 ng of this plasmid-DNA containing the VK-library (XhoI-BstEII digested; large fragment) were ligated with 900 ng of the heavy chain V-fragments (XhoI-BstEII digested) and again transformed into two 300 µl aliquots of electrocompetent E.coli XL1 Blue cells by electroporation (2.5 kV, 0.2 cm gap cuvette, 25 µ FD, 200 Ohm) resulting in a total V_H-V_K scFv (single chain variable fragment) library size of 4×10^8 independent clones.
After one hour of phenotype expression, positive transformation was selected by carbenicilline resistance.
After this adaptation, these clones were infected with an infectious dose of 1×10^{12} particles of the helper phage VCSM13 resulting in the production and secretion of filamentous phages, each of them containing single stranded pComb3H5BHis-DNA encoding a murine scFv-fragment and displaying the corresponding scFv-protein fused to the N2 domain on the phage surface as a translational fusion to phage coat protein III (phage display, see Figure 6.2).

This phage library carrying the cloned scFv-repertoire was harvested from the culture supernatant by PEG8000/NaCl precipitation and centrifugation, re-dissolved in TBS/1%BSA and incubated with recombinant soluble 17-1A immobilized on 96 well ELISA plates. Soluble 17-1A was prepared as described (Mack, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 7021-7025. Phage particles expressing N2-fused scFv-fragments that did not specifically bind to the target antigen were eliminated by up to ten washing steps with TBS/Tween. Binding entities were eluted by using HCl-Glycine pH 2.2 and after neutralization with 2 M Tris pH 12, the eluat was used for infection of a new uninfected E.coli XL1 Blue culture. Cells successfully transduced with a pComb phagmid copy, encoding a murine scFv-fragment, were again selected for carbenicilline resistance and subsequently infected with VCMS13 helper phage to start the second round of antibody display and in vitro selection.

After five rounds of phage-production and subsequent selection for antigen-binding scFv-displaying phages, plasmid DNA from E.coli cultures was isolated corresponding to 3, 4 and 5 rounds of panning as well as to the unselected repertoire prior to the first round of panning.

For the production of soluble scFv-antibody-fragments that carry the N2-domain at their N-terminus, the DNA fragment encoding the CT-domain of the geneIII-product was excised from the plasmids (SpeI/NheI), thus destroying the translational fusion anchoring the scFv-fragment to the phage surface. After religation this pool of plasmid DNA was transformed into 100 µl heat shock competent E.coli XL1 Blue cells and plated on Carbenicilline LB-Agar. Single colonies were grown in 10 ml LB-Carbenicilline-cultures/20 mM $MgCl_2$ and scFv-expression was induced after six hours by adding Isopropyl-β-D-thiogalactosid (IPTG) to a final concentration of 1 mM.

This in vitro selection procedure as well as the periplasmic expression of soluble antibody fragments was carried out according to Burton, Proc. Natl. Acad. Sci. USA 88 (1991), 10134-10137.

These cells were harvested after 20 hours by centrifugation and through four rounds of freezing at –70°C and thawing at 37°C the outer membrane of the bacteria was destroyed by temperature shock so that the soluble periplasmatic proteins including the N2-scFv fusion-proteins were released into solution. After elimination of intact cells and cell-debris by centrifugation, the supernatant was tested by ELISA for 17-1A-binding N2-scFv-fusion-proteins.

Detection of N2-scFv-fragments bound to immobilized soluble 17-1A anigen was carried out using an anti-His-tag antibody (1µg/ml PBS) detected with horse radish peroxidase conjugated polyclonal anti mouse antibody (1µg/ml PBS). The signal was developed by adding ABTS substrate solution, as described in Example 8, and detected at a wavelength of 405 nm.

In contrast to clones prior to antigen selection many clones obtained after 3, 4 and 5 rounds of panning showed 17-1A-binding activity as shown in Figure 6.2.

The DNA-sequence of the V_H- and V_K-regions of some positive clones (3-1; 3-5; 3-8; 4-1; 4-4; 4-7; 5-3; 5-10 and 5-13) was determined but none of the clones turned out to have identical V_H and VK DNA-sequence combinations (Figures 6.3-6.10 and 7).

Unless otherwise stated, the procedures used followed that described in Sambrook, Molecular Cloning, 'A Laboratory Manual', 2nd Edition, Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY (1989).

Example 7: Cloning of bifunctional CD80-anti-17-1A single-chain constructs by using the scFv-antibody-fragments generated by the method of the present invention

The following nine 17-1A-specific scFv constructs obtained by the procedure described in Example 6

17-1A 3-1	in p-Comb3H-5B-His
17-1A 3-5	in p-Comb3H-5B-His
17-1A 3-8	in p-Comb3H-5B-His
17-1A 4-1	in p-Comb3H-5B-His
17-1A 4-4	in p-Comb3H-5B-His
17-1A 4-7	in p-Comb3H-5B-His
17-1A 5-3	in p-Comb3H-5B-His
17-1A 5-10	in p-Comb3H-5B-His
17-1A 5-13	in p-Comb3H-5B-His

were subcloned into the vector pEF-DHFR for stable expression in CHO-cells. In this step the N2-domain was replaced by the two extracellular domains of human CD80 (= B7-1).

For this purpose the vector pEF-DHFR + CTI + CD80 + scFv VD4.5VK8 described in Example 3 was cleaved the same way as the fragments derived from pComb3H5BHis clones 3-1 to 5-13 using the restriction enzymes BspEI and NotI according to standard procedures (Sambrook, Molecular Cloning; A

Laboratory Manual, Second Edition, Cold Spring Harbour Laboratory Press, Cold Spring Habour, NY (1989)).

Both, vector and fragments were isolated on a 1% agarose gel, the specific bands were eluted using a commercial gel elution kit (Qiagen). After ligation DNA was transformed into the E.coli strain XL-1 blue by the standard heat shock method (Sambrook, Molecular Cloning; A Laboratory Manual, Second Edition, Cold Spring Harbour Laboratory Press, Cold Spring Habour, NY (1989)).

Positive clones were detected by PCR-based colony screening with the following primers:

5' B7-1 5'- GCA GAA TTC ACC ATG GGC CAC ACA CGG AGG CAG-3'
3' mu VK 5'-TGG TGC ACT AGT CGT ACG TTT GAT CTC AAG CTT GGT CCC-3'

One clone of each construct was grown to a 200 ml LB culture in the presence of 50 µg/ml ampicillin. Plasmid-DNA was purified with the commercially available Mega Prep kit (Qiagen) and linearized by the restriction enzyme Nde I. Finally these linearized plasmid-DNAs were transfected into dihydrofolate-reductase (DHFR) deficient CHO cells by electroporation at 260 V and 960 µFD as described (Mack, Proc. Natl. Acad. Sci. U.S.A. 92 (1995), 7021-7025).
Primary selection was carried out in nucleoside-free alpha MEM culture medium supplemented with 10% dialysed FCS as described (Kaufmann, Methods Enzymol. 185 (1990), 537-566).
The expression of these constructs was increased by gene amplification induced by the addition of the DHFR-inhibitor methotrexate (MTX) to a final concentration of 20nM as described (Kaufmann, Methods Enzymol. 185 (1990), 537-566).

Example 8: ELISA-analysis of bifunctional CD80-anti-17-1A-scFv-constructs produced by the method of the present invention

The culture supernatants of these transfected cell-lines derived from primary selection and first amplification step were tested by ELISA. Therefore recombinant soluble 17-1A (Mack, Proc. Natl. Acad. Sci. U.S.A. 92 (1995), 7021-7025) was coated to 96 well U-bottom ELISA plates (Nunc maxisorb) (50µg/ml 50µl/well) in phosphate buffered saline (PBS). Coating was performed overnight at 4°C, blocking was performed with 3% bovine serum albumin (BSA) in PBS for one hour at room temperature. Antibody constructs as culture supernatants from primary selection (PS) (Figure 8.1 and 8.2) and the first amplification step (1. Amp.) (Figure 8.3 and 8.4), respectively, were added and incubated for one hour at room temperature at different dilutions made in PBS containing 1% BSA.

Bound bifunctional antibody constructs were detected by a CD80-specific monoclonal antibody (Immunotech., Cat. No. 1449) diluted 1:1000 in PBS 1%BSA. After three times of washing with PBS 0,05% Tween20, a polyclonal peroxidase-conjugated Goat Anti-Mouse IgG-antibody (Fc-specific) was added and incubated at room temperature for one hour. After four times of washing with PBS 0,05% Tween20, the ELISA was finally developed by adding the following substrate solution: 22 mg ABTS (2,2 Azino-bis (3-Ethylbenzthiazoline-6 Sulfonic Acid) Diammonium salt) was dissolved in 10 ml 0,1 M citrat buffer pH 5,1 containing 2,3 mg Sodium perborate tetrahydrate. For negative controls, the plates were incubated with PBS instead of bifunctional antibody constructs. The coloured precipitate was measured at 405 nm using an ELISA-reader.

As shown in Figures 8.1 and 8.2, all clones proved to bind to the 17-1A-antigen with varying binding intensities.

Example 9: Flowcytometry analysis of bifunctional CD80-anti-17-1A-scFv-contructs produced by the method of the present invention

The culture supernatants from the first gene amplification step each containing one of the nine 17-1A-specific bifunctional CD80-scFv-constructs (Example 7) were tested on 17-1A-transfected CHO-cells by flow cytometry. These transfected cell-lines were generated by subcloning of a DNA-fragment encoding the complete amino acid sequence of the 17-1A-antigen also known as GA733-2 (Szala, Proc. Natl. Acad. Sci. U.S.A. 87 (1990), 3542-3546), into the eukaryotic expression vector pEF-DHFR according to standard procedures (Sambrook, Molecular Cloning; A Laboratory Manual, Second Edition, Cold Spring Harbour Laboratory Press, Cold Spring Habour, NY (1989)); linearization of the resulting plasmid with the restriction enzyme Nde I and subsequent stable transfection into DHFR-deficient CHO cells was performed as described in Example 7. The expression of transmembrane 17-1A was increased by stepwise gene amplification induced by subsequent addition of increasing concentrations of the DHFR-inhibitor Methotrexat (MTX) to a final concentration of 500nM, with the concentration steps in between being 20nM and 100nM (Kaufmann, Methods Enzymol. 185 (1990), 537-566).

These cells were tested for membrane expression of 17-1A by flow cytometry using the 17-1A-specific monoclonal antibody M79 (Göttlinger, Int. J. Cancer 38 (1986), 47-53) at a concentration of 10 µg/ml followed by a polyclonal Goat Anti Mouse IgG + IgM (H+L) antibody diluted 1:100 in PBS. As negative control untransfected CHO cells were used whereas the 17-1A-positive human gastric cancer cell-line Kato, obtained from ATCC served as positive control. Results are shown in Figure 9.2..

Binding of the bifunctional CD80-scFv-constructs produced by the method of the present invention on 17-1A-positive cells was analysed as follows:

For this purpose adherent untransfected and 17-1A-transfected CHO-cells were detached using PBS containing 0,05% Trypsine, respectively. $5x10^5$ cells were incubated for 30 minutes on ice in 50 µl culture supernatant containing the corresponding bifunctional construct undiluted (Figure 9.1). Bound bifunctional CD80-scFv-constructs were detected by a monoclonal anti-CD80 antibody (Immunotech. Cat. No: 1449) diluted 1:20 in 50 µl PBS. Incubation conditions were the same as above. Bound CD80-antibody was finally detected by a fluorescein conjugated polyclonal Goat Anti-Mouse IgG + IgM (H+L) antibody diluted 1:100 in PBS. Incubation was again carried out for 30 minutes on ice. For the fixation of fluorescein-labeled cells 1% paraformaldehyd in PBS was used.

As first negative control untransfected CHO-cells were used. The second negative control contained 17-1A-transfected cells that were incubated with PBS instead of bifunctional CD80-scFv-constructs. Staining with the monoclonal antibody M79 (Göttlinger, Int. J. Cancer 38 (1986), 47-53) was used as positive control.

Cells were analysed by flow cytometry on a FACS scan (Becton Dickenson). FACS staining and measuring of the fluorescence intensity were performed as described in Current Protocols in Immunology (Coligan, Kruisbeek, Margulies, Shevach and Strober, Wiley-Interscience, 1992)

As shown in Figure 9.1 all nine bifunctional CD80-scFv-constructs bound to the 17-1A-antigen on the cell surface thus confirming the ELISA- results of Example 8.

Table 1: Primer sets for the amplification of the VH- and VK-DNA-fragments (5' to 3')

murine V heavy chain:

5'primer	
MVH1	5'- (GC)AGGTGCAGCTCGAGGAGTCAGGACCT-3'
MVH2	5'-GAGGTCCAGCTCGAGCAGTCTGGACCT-3'
MVH3	5'-CAGGTCCAACTCGAGCAGCCTGGGGCT-3'
MVH4	5'-GAGGTTCAGCTCGAGCAGTCTGGGGCA-3'
MVH5	5'-GA(AG)GTGAAGCTCGAGGAGTCTGGAGGA-3'
MVH6	5'-GAGGTGAAGCTTCTCGAGTCTGGAGGT-3'
MVH7	5'-GAAGTGAAGCTCGAGGAGTCTGGGGGA-3'
MVH8	5'-GAGGTTCAGCTCGAGCAGTCTGGAGCT-3'
3'primer	
MUVHBstEII	5'-TGAGGAGACGGTGACCGTGGTCCCTTGGCCCCAG-3'

murine V kappa chain:

5'primer	
MUVK1	5'-CCAGTTCCGAGCTCGTTGTGACTCAGGAATCT-3'
MUVK2	5'-CCAGTTCCGAGCTCGTGTTGACGCAGCCGCCC-3'
MUVK3	5'-CCAGTTCCGAGCTCGTGCTCACCCAGTCTCCA-3'
MUVK4	5'-CCAGTTCCGAGCTCCAGATGACCCAGTCTCCA-3'
MUVK5	5'-CCAGATGTGAGCTCGTGATGACCCAGACTCCA-3'
MUVK6	5'-CCAGATGTGAGCTCGTCATGACCCAGTCTCCA-3'
MUVK7	5'-CCAGTTCCGAGCTCGTGATGACACAGTCTCCA-3'
3'primer	
MUVKHindIII/BsiWI	5'-TGGTGCACTAGTCGTACGTTTGATCTCAAGCTTGGTCCC-3'

CLAIMS

1. A method of identifying a binding site domain having the capacity of binding to a predetermined epitope when positioned C-terminal of at least one further domain in a recombinant bi- or multivalent polypeptide comprising the steps of

   (a) testing a panel of binding site domains displayed on the surface of a biological display system as part of a fusion protein for binding to a predetermined epitope, wherein said fusion protein comprises an additional domain positioned N-terminal of said binding site domain and an amino acid sequence that mediates anchoring of the fusion protein to the surface of said display system; and

   (b) identifying a binding site domain that binds to said predetermined epitope.

2. The method of claim 1, wherein said binding site domain and said additional domain are linked by a polypeptide linker disposed between said binding site and said additional domain, wherein said polypeptide linker comprises plural, hydrophilic, peptide-bonded amino acids and connects the N-terminal end of said binding site domain and the C-terminal end of said additional domain.

3. The method of claim 1 or 2, wherein said binding site domain is a pair of V_H-V_L, V_H-V_H or V_L-V_L domains.

4. The method of any one of claims 1 to 3 wherein said display system is a filamentous phage produced by bacteria transfected therewith, a baculovirus expression system, a ribosome based expression system, a bacteriophage lambda display system or a bacterial surface expression system.

5. The method of claim 4 comprising, prior to step (a), the further step of

(a") transfecting bacteria with recombinant vectors encoding said fusion proteins.

6. The method of any one of claims 1 to 5 comprising, prior to step (a"), the further step of

(a') cloning a panel of nucleic acid molecules encoding said binding site domains into a vector.

7. The method of claim 6, wherein said panel of nucleic acid molecules is derived from immune competent cells of a mammal, fish or bird.

8. The method of any one of claims 1 to 7, wherein said additional domain comprises at least 9 amino acids.

9. The method of claim 8, wherein said additional domain is or is derived from the N2-domain of the gene III product of filamentous phage.

10. The method of any one of claims 1 to 9, wherein said sequence that mediates said anchoring is or is derived from the C-terminal CT-domain of the gene III product of filamentous phage.

11. The method of any one of claims 1 to 8, wherein said bi- or multivalent polypeptide is a bi- or multifunctional polypeptide.

12. The method of claim 9, wherein said at least one further domain comprises polypeptide selected from the group consisting of effector proteins having a conformation suitable for biological activity, amino acid sequences capable of sequestering an ion, and amino acid sequences capable of selective binding to a solid support.

13. The method of claim 12 wherein said effector protein is an enzyme, toxin, receptor, binding site, biosynthetic antibody binding site, growth factor, cell-differentiation factor, lymphokine, cytokine, hormone, a remotely detectable moiety, or anti-metabolite.

14. The method of claim 12 wherein said sequence capable of sequestering an ion is calmodulin, methallothionein, a fragment thereof, or an amino acid sequence rich in at least one of glutamic acid, aspartic acid, lysine, and arginine.

15. The method of claim 12 wherein said polypeptide sequence capable of selective binding to a solid support is a positively or negatively charged amino acid sequence, a cysteine-containing amino acid sequence, streptavidin, or a fragment of Staphylococcus protein A.

16. The method of claim 13, wherein said receptor is a co-stimulatory surface molecule important for T-cell activation or comprises an epitope binding site or a hormone binding site.

17. The method of claim 16, wherein said co-stimulatory surface molecule is CD80 (B7-1).

18. The method of claim 17, wherein said epitope binding site is embedded in a pair of V_H-V_L, V_H-V_H or V_L-V_L domains.

19. The method of any one of claims 3 to 18, wherein said pair of domains are connected by a flexible linker, preferably by a polypeptide linker disposed between said domains, wherein said polypeptide linker comprises plural, hydrophilic, peptide-bonded amino acids of a length sufficient to span the distance between the C-terminal end of one of said domains and the N-terminal end of the other of said domains

when said fusion protein assumes a conformation suitable for binding when disposed in aqueous solution.

20. The method of any one of claims 1 to 19, wherein the identification of said binding site domain comprises the steps of

(b') removing said amino acid sequence that mediates anchoring of the fusion protein to the surface of a phage from said fusion protein;

(b") periplasmatically expressing the nucleic acid molecules encoding the remainder of said fusion protein in bacteria; and

(b"') verifying whether said binding site domain binds to said predetermined epitope.

21. A recombinant vector as defined in anyone of claims 1 to 20.

22. A host cell harboring and capable of suppressing the recombinant vector of claim 21.

23. Kit comprising

(a) a recombinant vector of claim 21 or a panel of recombinant vectors encoding a panel of fusion proteins as defined in any one of claims 1 to 20; and/or

(b) a host cell of claim 22 or a bacterial library transfected with a panel of vectors as defined in (a).

24. A binding site domain or fusion protein obtainable by the method of any one of claims 1 to 20.

25. The binding site domain or fusion proteins of claim 24, wherein said binding site domain comprises at least one complementarity determining region (CDR) of the scFv fragment shown in any one of figures 6.3 to 6.10 and 7.

26. A polypeptide or an antibody comprising at least one binding site domain or fusion protein of claim 24 or 25.

27. The polypeptide or antibody of claim 26 having the amino acid sequence as depicted in any one of figures 6.3 to 6.10 and 7.

28. Polynucleotides which upon expression encode the polypeptide or antibody of claim 26 or 27.

29. A cell transfected with a polynucleotide of claim 28.

30. A process for the preparation of a polypeptide or antibody of claim 26 or 27 comprisind cultivating a cell of claim 29 under conditions suitable for the expression of the polypeptide and isolating the polypeptide from the cell culture medium.

31. A pharmaceutical composition containing a polypeptide or antibody of claim 26 or 27 and optionally a pharmaceutically acceptable carrier.

32. A diagnostic composition comprising the polypeptide or antibody of claim 26 or 27 and optionally suitable means for detection.

ABSTRACT

The present invention relates to a method of identifying binding site domains that retain the capacity of binding to an epitope when positioned C-terminal of at least one further domain in a recombinant bi- or multivalent polypeptide. The present invention further relates to a kit comprising components such as panels of recombinant vectors of bacterial libraries transfected with a panel of recombinant vectors which is useful in carrying out the method of the invention. Furthermore, binding site domains and fusion proteins obtainable by the method of the invention as well as antibody-like molecules comprising such domains and proteins are described. Furthermore, pharmaceutical and diagnostic compositions containing the above-described fusion proteins and polypeptides are provided.

Figure 1.1

Recombinant bifunctional single-chain protein

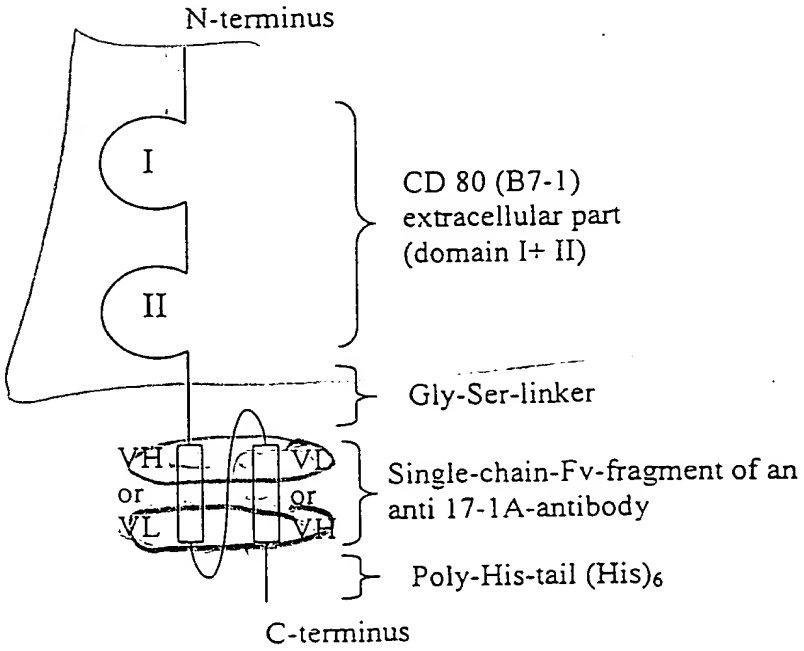


Figure 1.2 DNA-sequence designated CTI

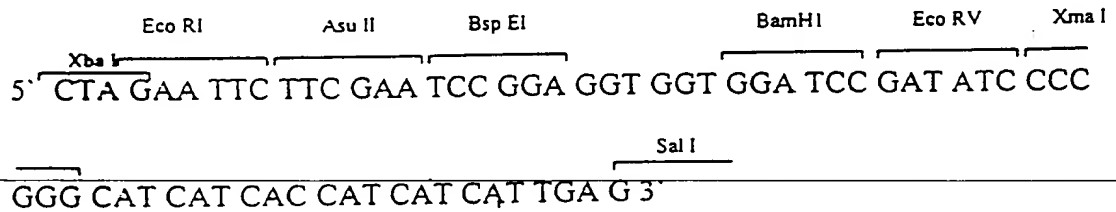


Figure 1.3 Design of various bifunctional CD80-scFv-constructs

Figure 1.3.1.

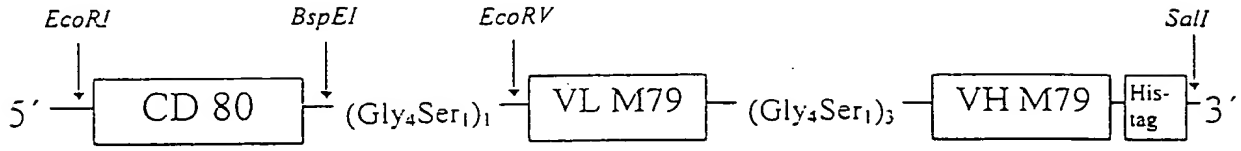


Figure 1.3.2.

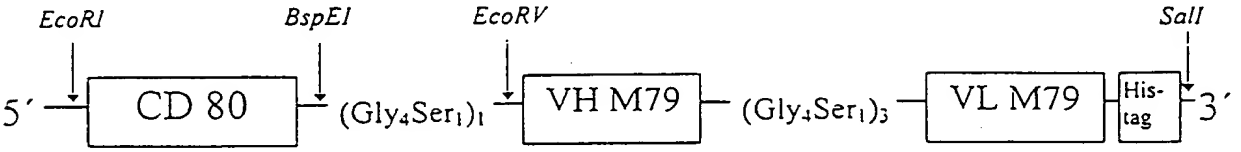


Figure 1.3.3.

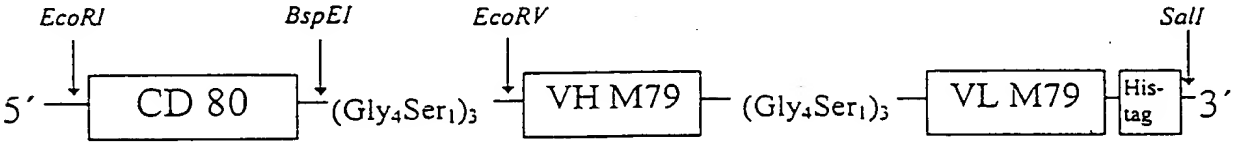


Figure 1.3.4.

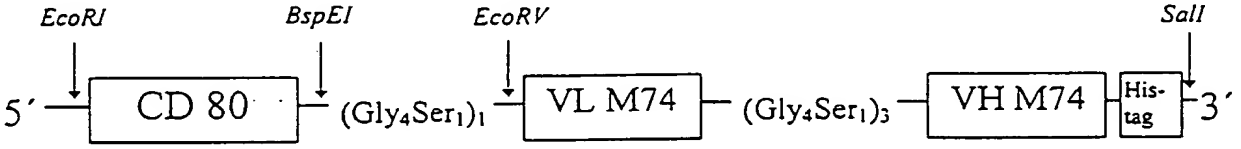


Figure 1.3.5.

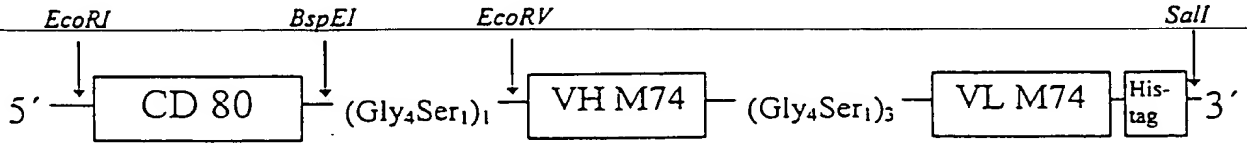


Figure 1.3.6.

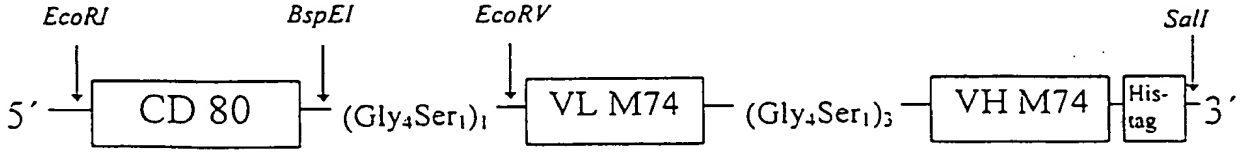


Figure 1.3.7

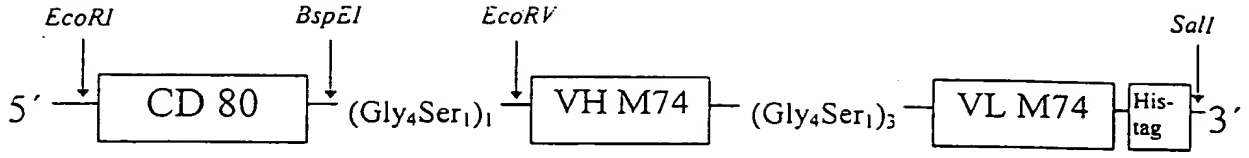


Figure 1.3.8.

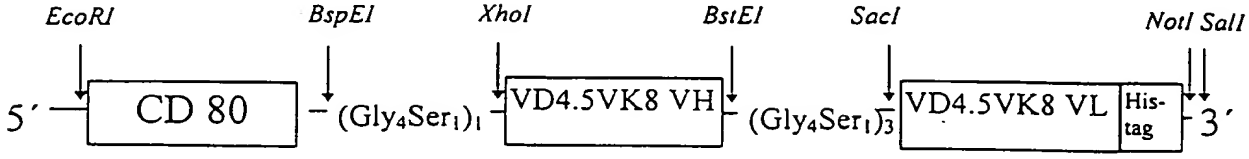


Figure 1.3.9.

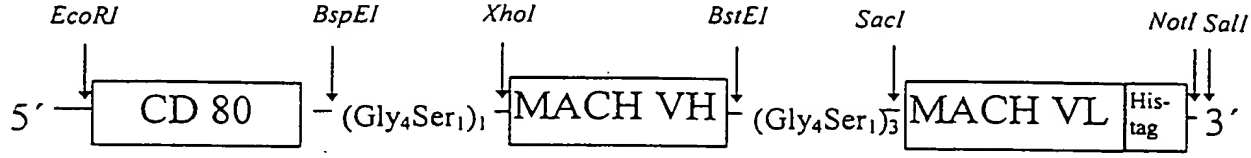


Figure 1.4

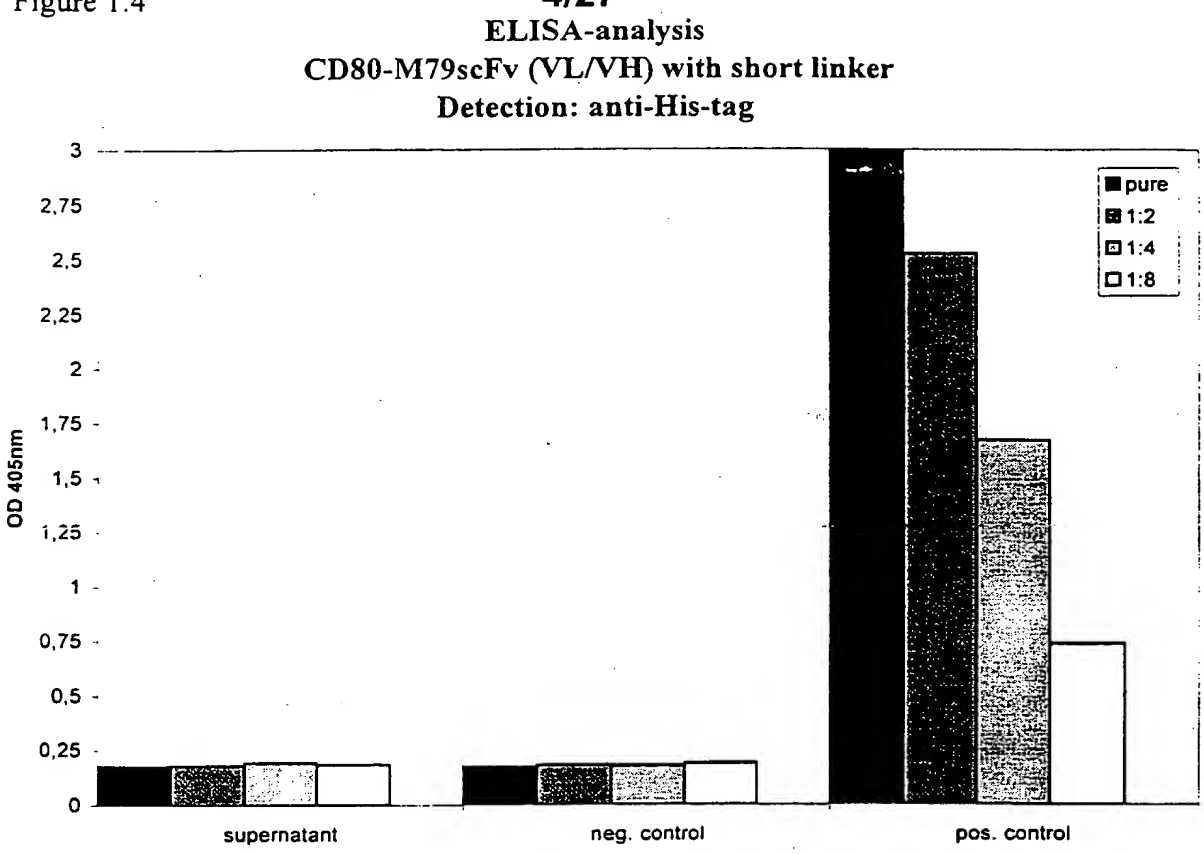


Figure 1.5

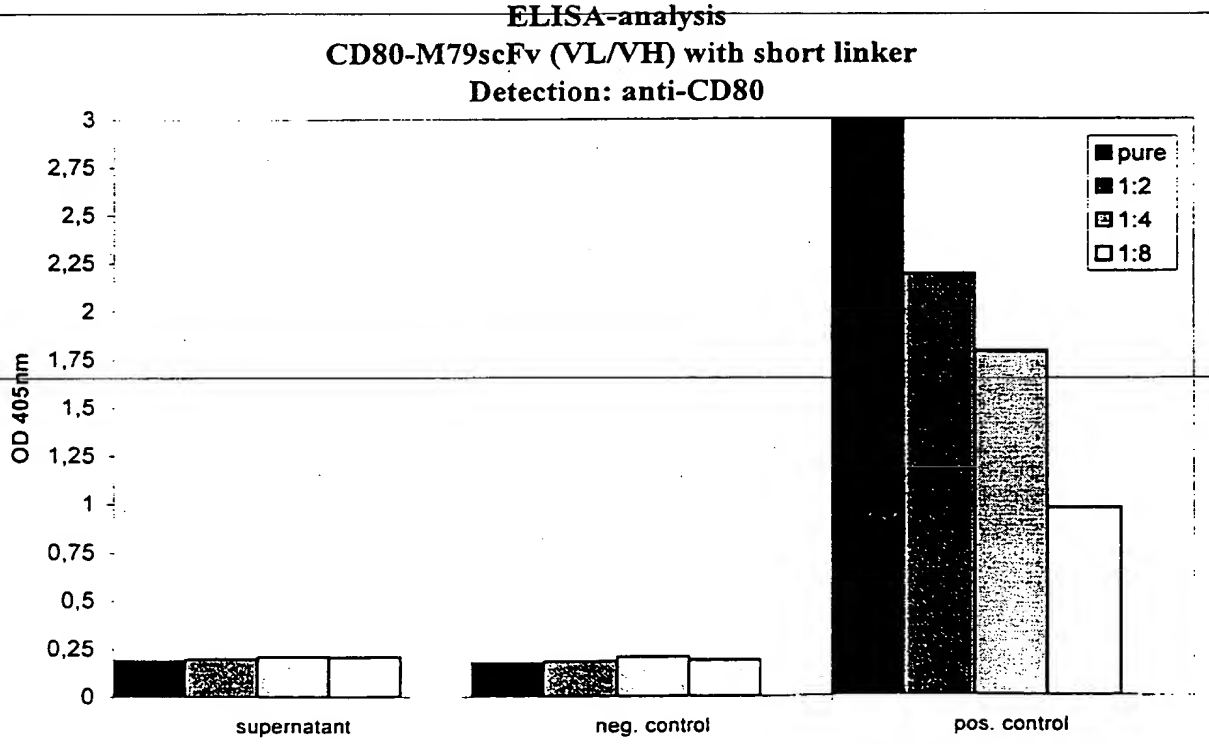


Figure 1.6

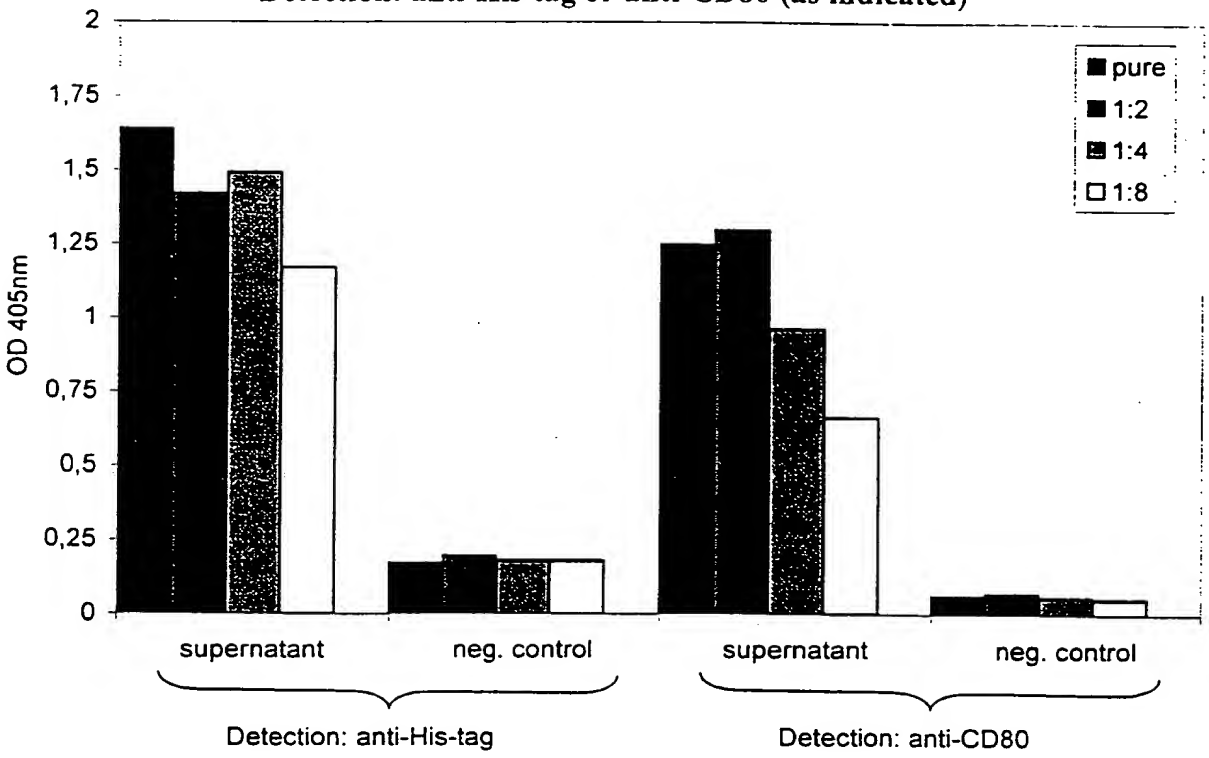


Figure 1.7

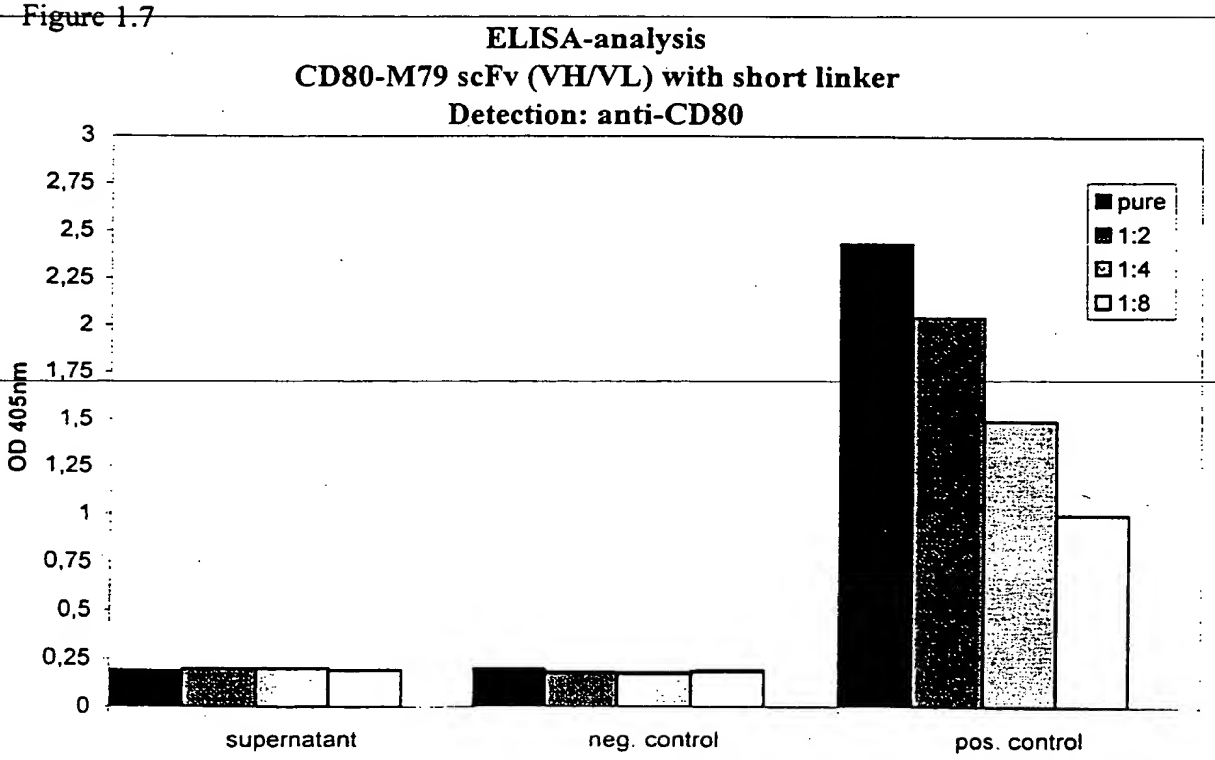


Figure 1.8 DNA-sequence of the double-stranded oligonucleotide designated ACCGS15BAM

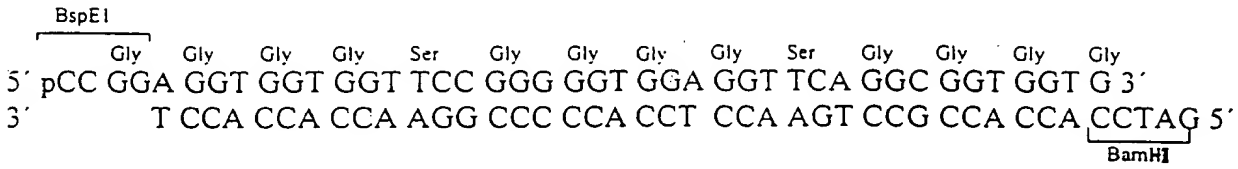


Figure 1.9

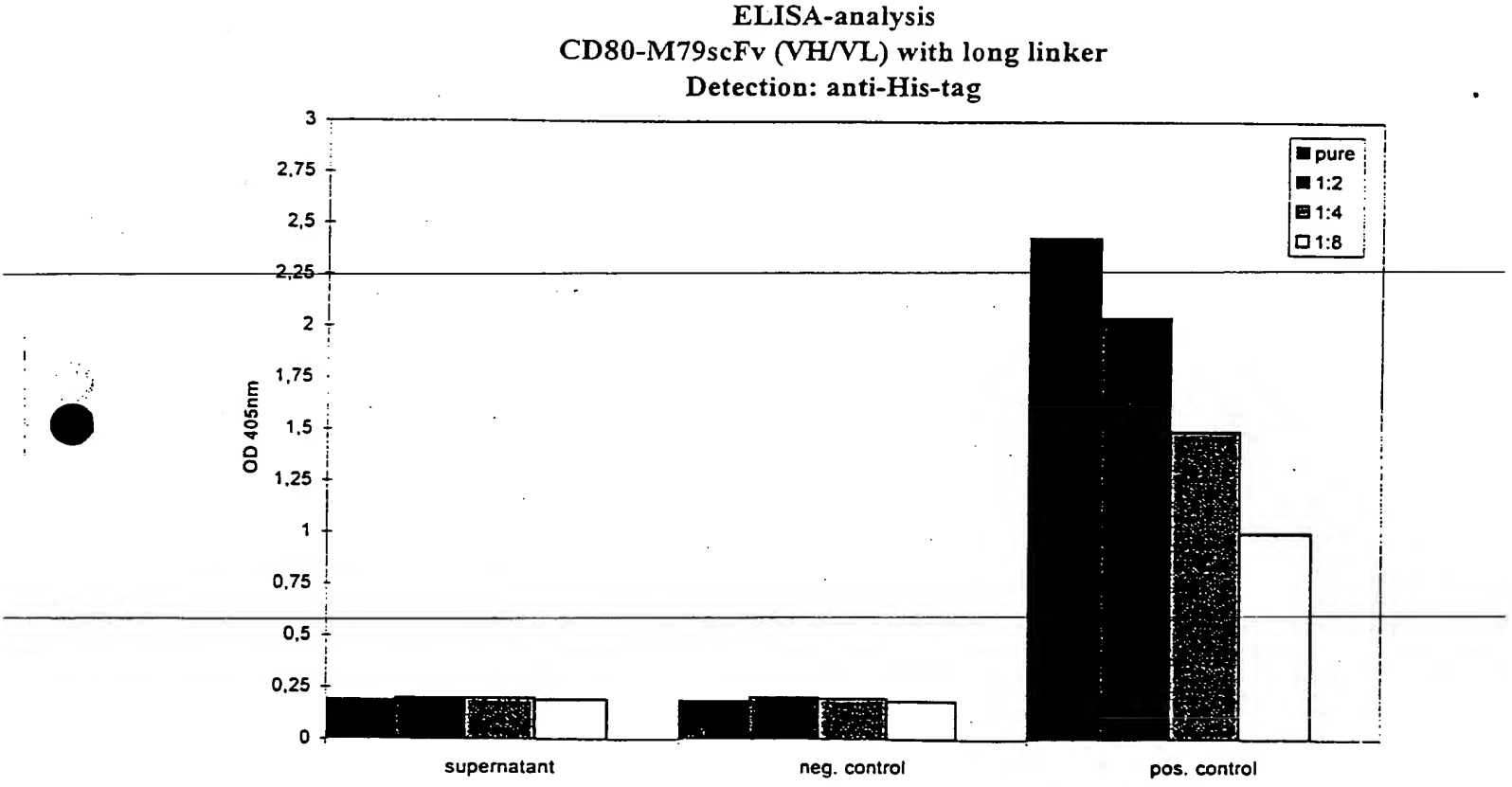


Figure 2.1

Elisa-analysis
CD80-M74 scFv with different linker lengths and either VL/VH- or VH/VL-domain arrangement (as indicated)
Detection: anti-His-tag

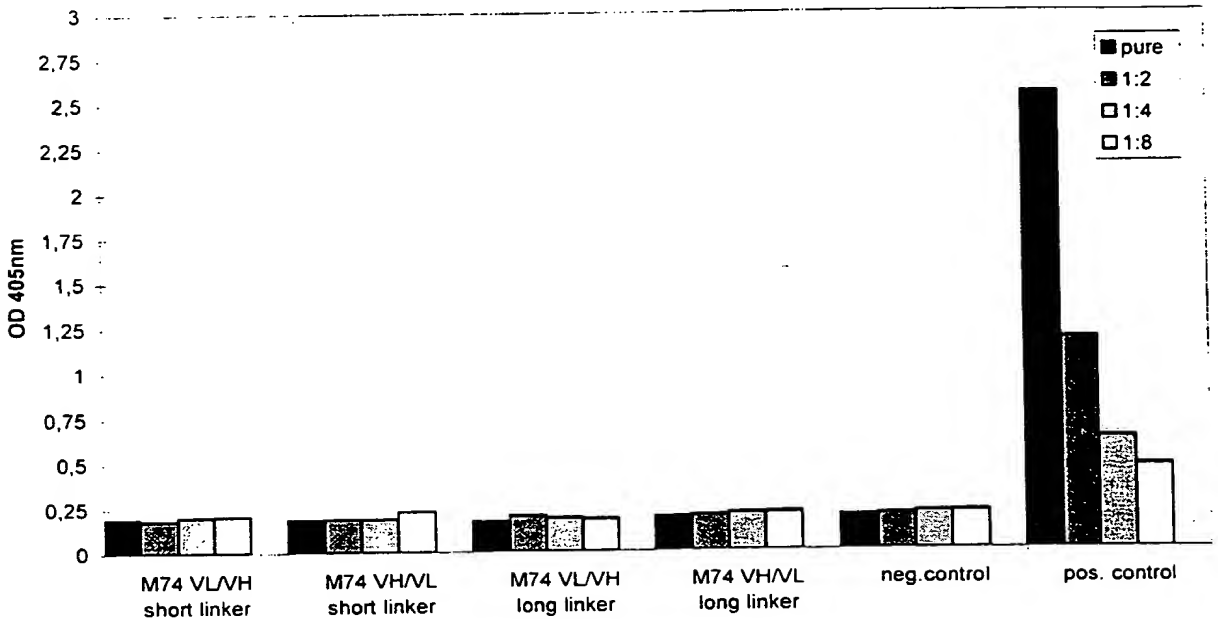


Figure 2.2

ELISA-analysis
CD80-M74 scFv with different linker lengths and either VL/VH- or VH/VL-domain arrangement (as indicated)
Detection: anti-CD80

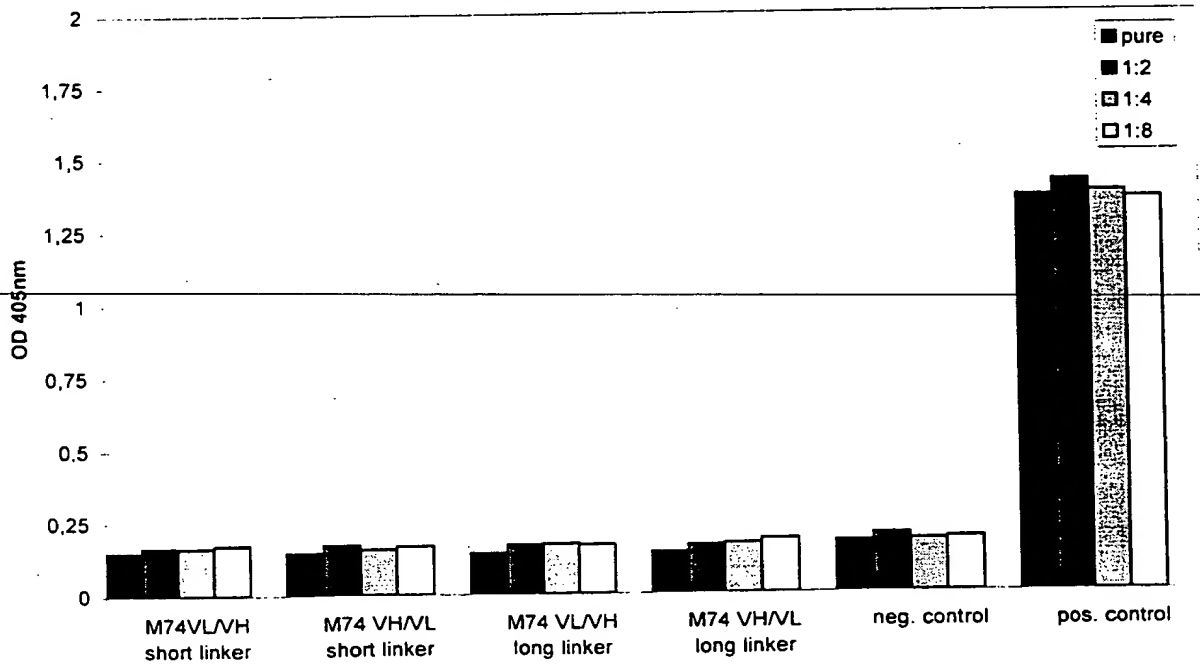
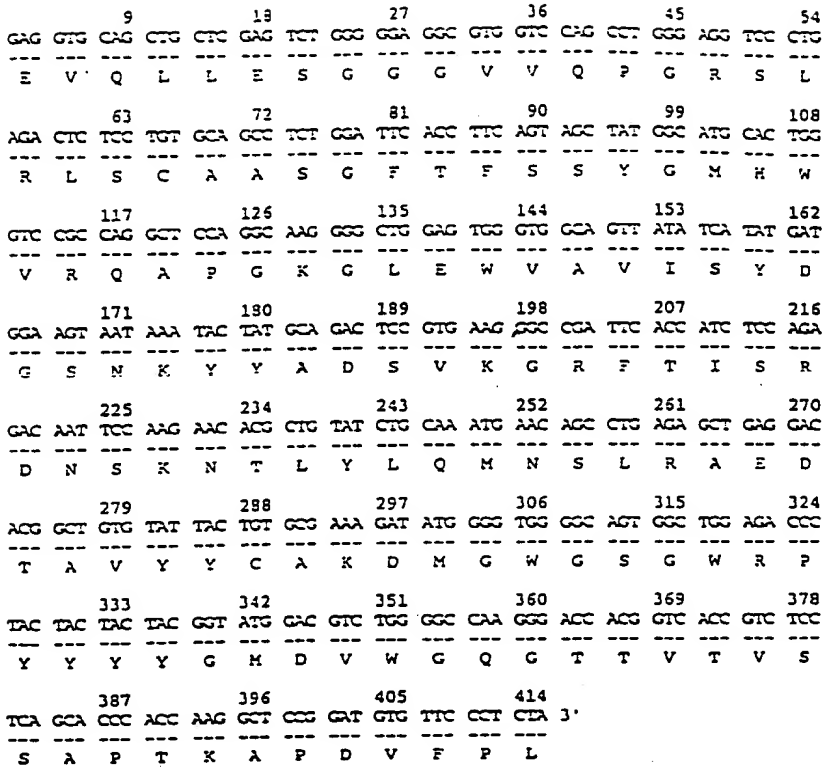


Figure 3.1



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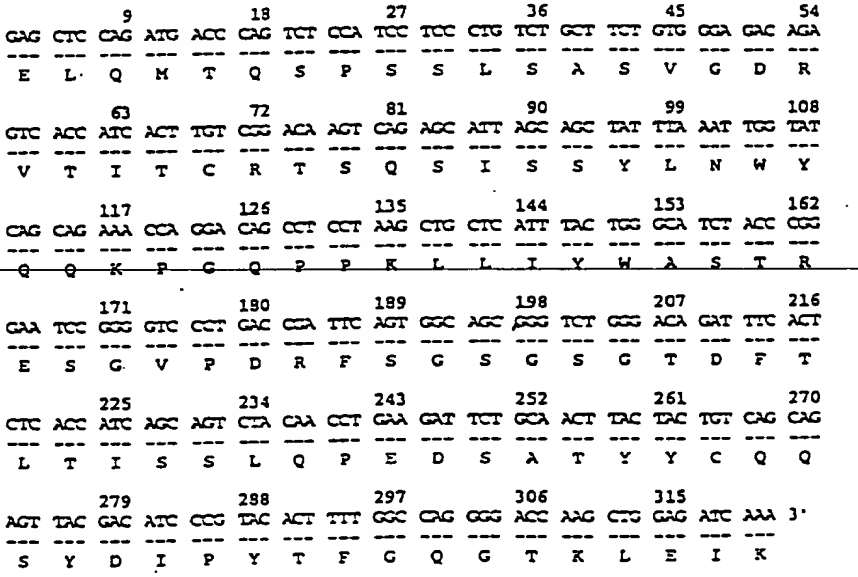

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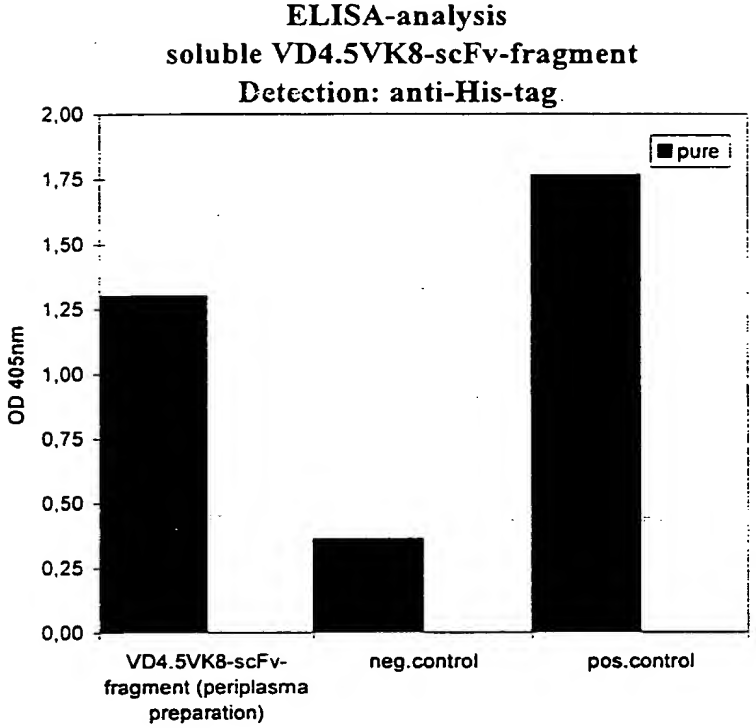


Figure 3.4 DNA-sequence designated L-F-NS3Frame

EcoRI

5´ CCG CTC TAG AAT TCC ACC ATG GGA TGG AGC TGT ATC ATC CTC TTC TTG

GTA GCA ACA GCT ACA GGT GTC CAC TCC GAC TAC AAA GAT GAT GAC GAT

EcoRV Eco47III NdeI

AAG GAT ATC TCC GGA GGT GGT GGT AGC GCT ATT CCA TAT GGA CGT CCC

XhoI Not I XbaI

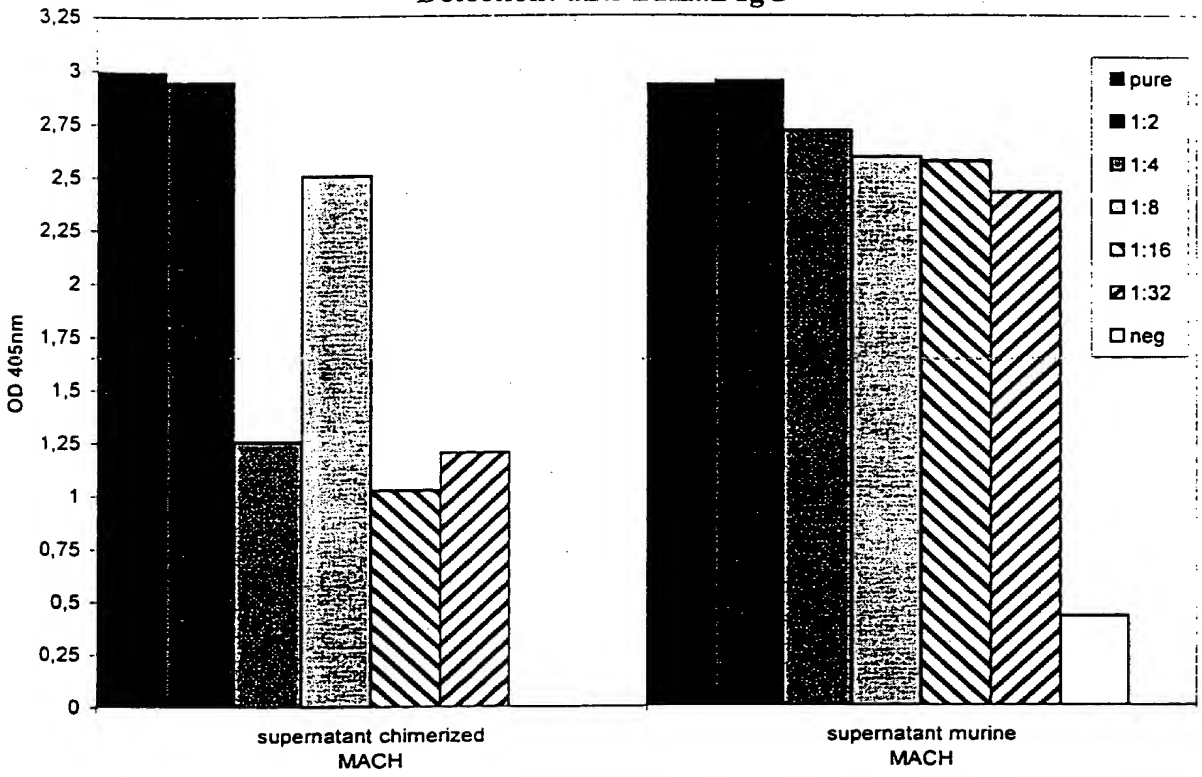
GCT CGA GGT CGT CCA TCA TCA CCA TCA TCA CTG AGC GGC CGC TCT AGA

Sal I

GTC GAC CTC 3`

Figure 4

ELISA-analysis
chimerized anti-17-1A antibody MACH
Detection: anti-human IgG



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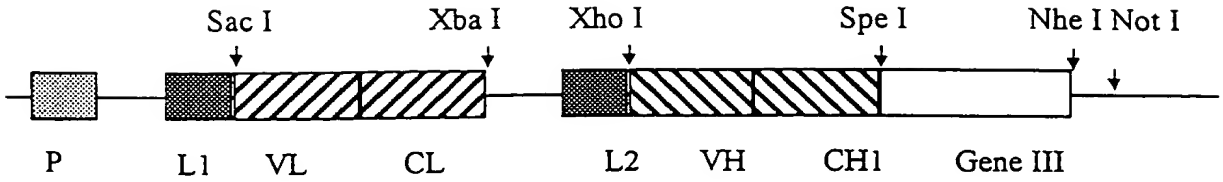

Figure 5.2

destroyed SacI — SalI

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G   Y   T   Y   I   N   P   L   D   G   T   Y   P   P   G   T   E   Q

AAC CCC GCT AAT CCT AAT CCT TCT CTT GAG GAG TCT CAG CCT CTT AAT ACT TTC
N   P   A   N   P   N   P   S   L   E   E   S   Q   P   L   N   T   F

ATG TTT CAG AAT AAT AGG TTC CGA AAT AGG CAG GGG GCA TTA ACT GTT TAT ACG
M   F   Q   N   N   R   F   R   N   R   Q   G   A   L   T   V   Y   T

GGC ACT GTT ACT CAA GGC ACT GAC CCC GTT AAA ACT TAT TAC CAG TAC ACT CCT
G   T   V   T   Q   G   T   D   P   V   K   T   Y   Y   Q   Y   T   P

GTA TCA TCA AAA GCC ATG TAT GAC GCT TAC TGG AAC GGT AAA TTC AGA GAC TGC
V   S   S   K   A   M   Y   D   A   Y   W   N   G   K   F   R   D   C

GCT TTC CAT TCT GGC TTT AAT GAG GAT CCA TTC GTT TGT GAA TAT CAA GGC CAA
A   F   H   S   G   F   N   E   D   P   F   V   C   E   Y   Q   G   Q

TCG TCT GAC CTG CCT CAA CCT CCT GTC AAT GCT TCC GGA GGT GGT GGA TCC GAG
S   S   D   L   P   Q   P   P   V   N   A   S   G   G   G   G   S

                XhoI          BstEII
GTG CAG CTG CTC GAG CCC GGT CAC CGT CTC CTC AGG TGG TGG TGG TTC TGG CGG

                                        SacI        SpeI
CGG CGG CTC CGG TGG TGG TGG TTC TGA GCT CGG GAC TAG T 3'
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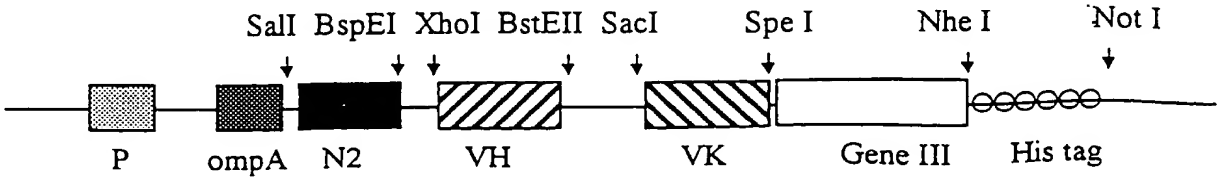
Figure 5.3



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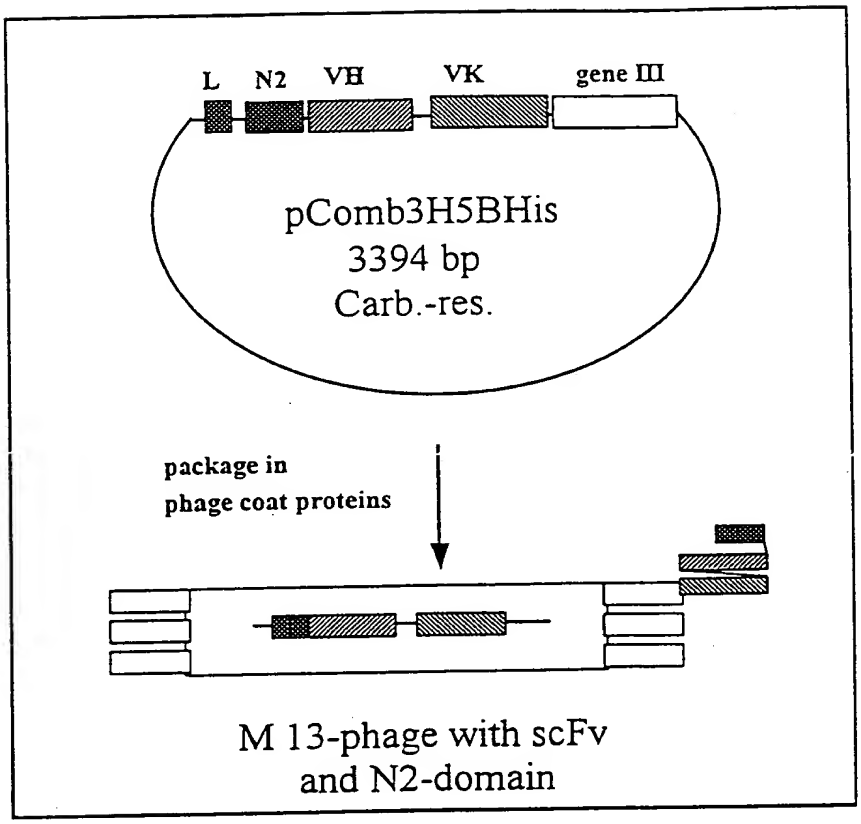

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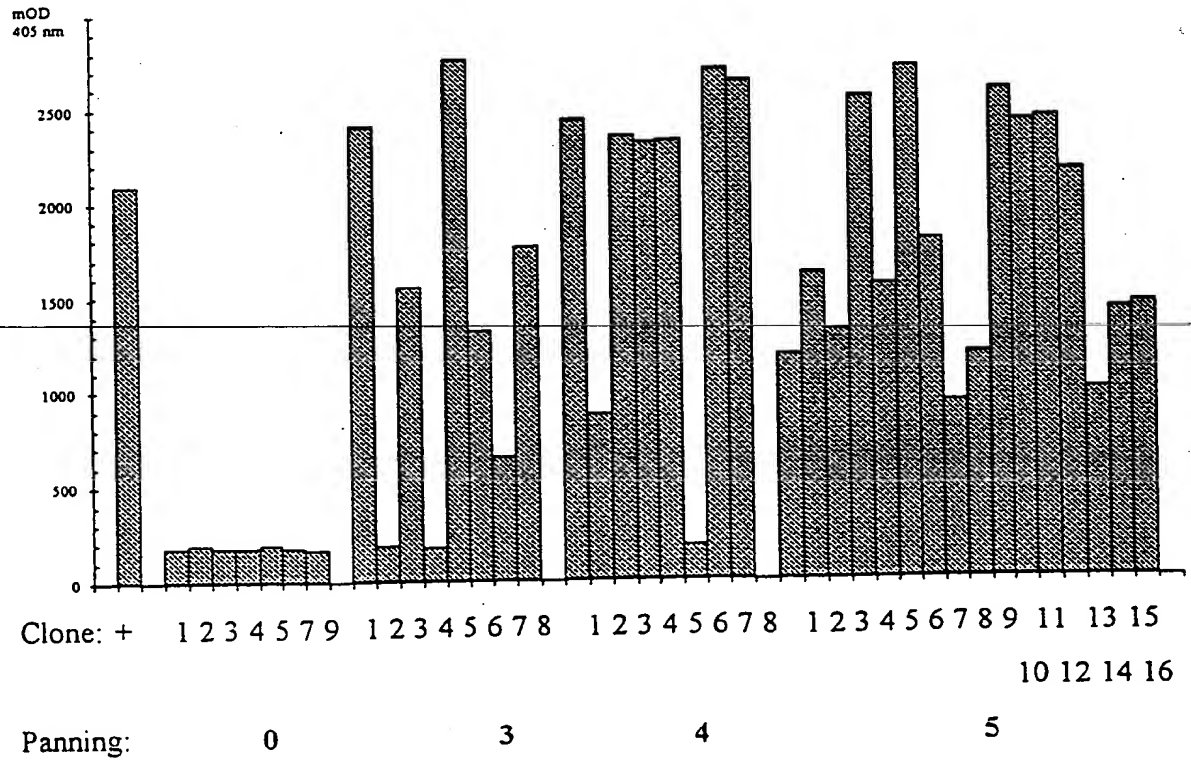

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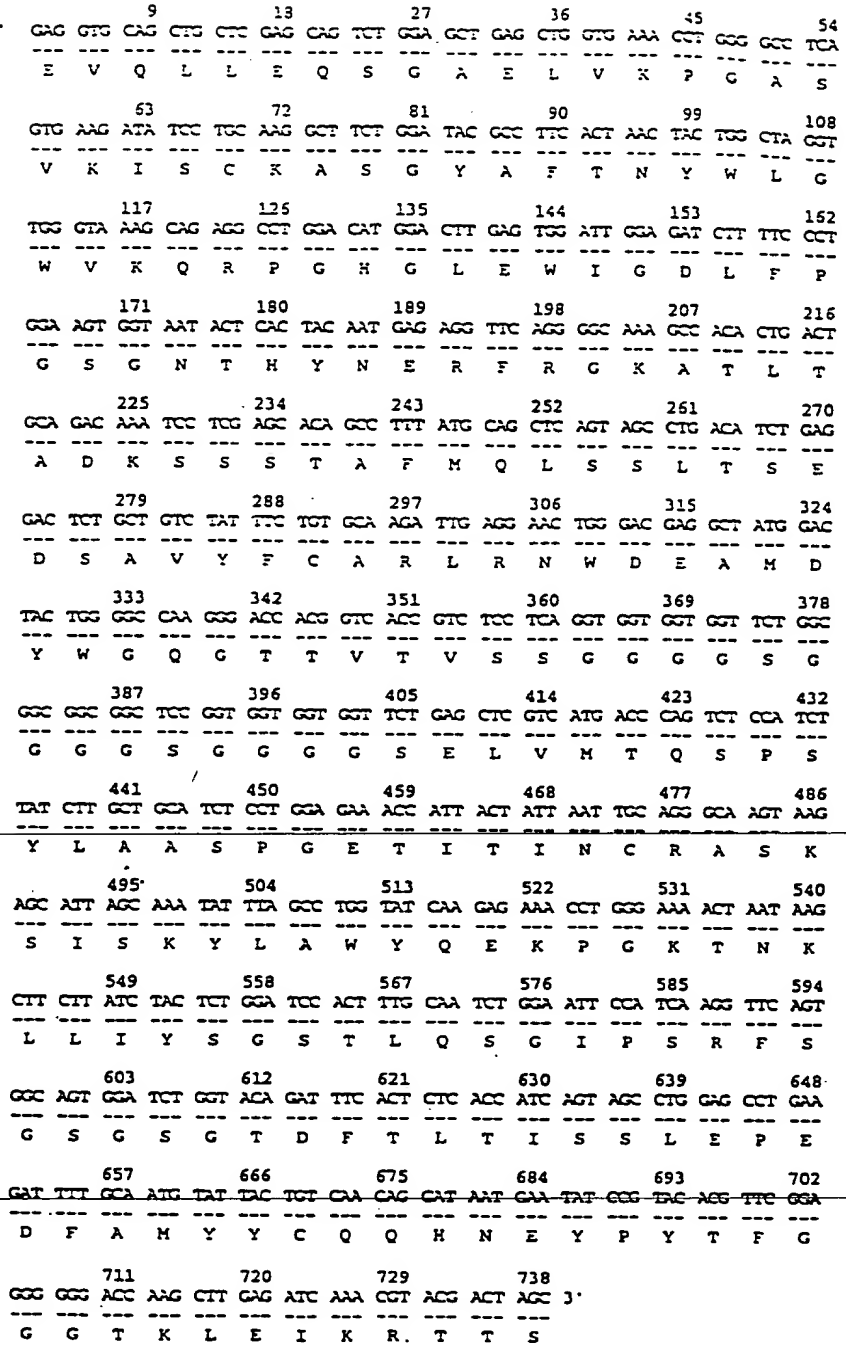
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TGG GTA AAG CAG AGG CCT GGA CAT GGA CTT GAG TGG ATT GGA GAT CTT TTC CCT
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G S G N T H Y N E R F R G K A T L T
225 234 243 252 261 270
GCA GAC AAA TCC TCG AGC ACA GCC TTT ATG CAG CTC AGT AGC CTG ACA TCT GAG
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Y W G Q G T T V T V S S G G G G S G
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G S G S G T D F T L T I S S L E P E
657 666 675 684 693 702
GAT TTT GCA ATG TAT TAC TGT CAA CAG CAT AAT GAA TAT CCG TAC ACG TTC GGA
D F A M Y Y C Q Q H N E Y P Y T F G
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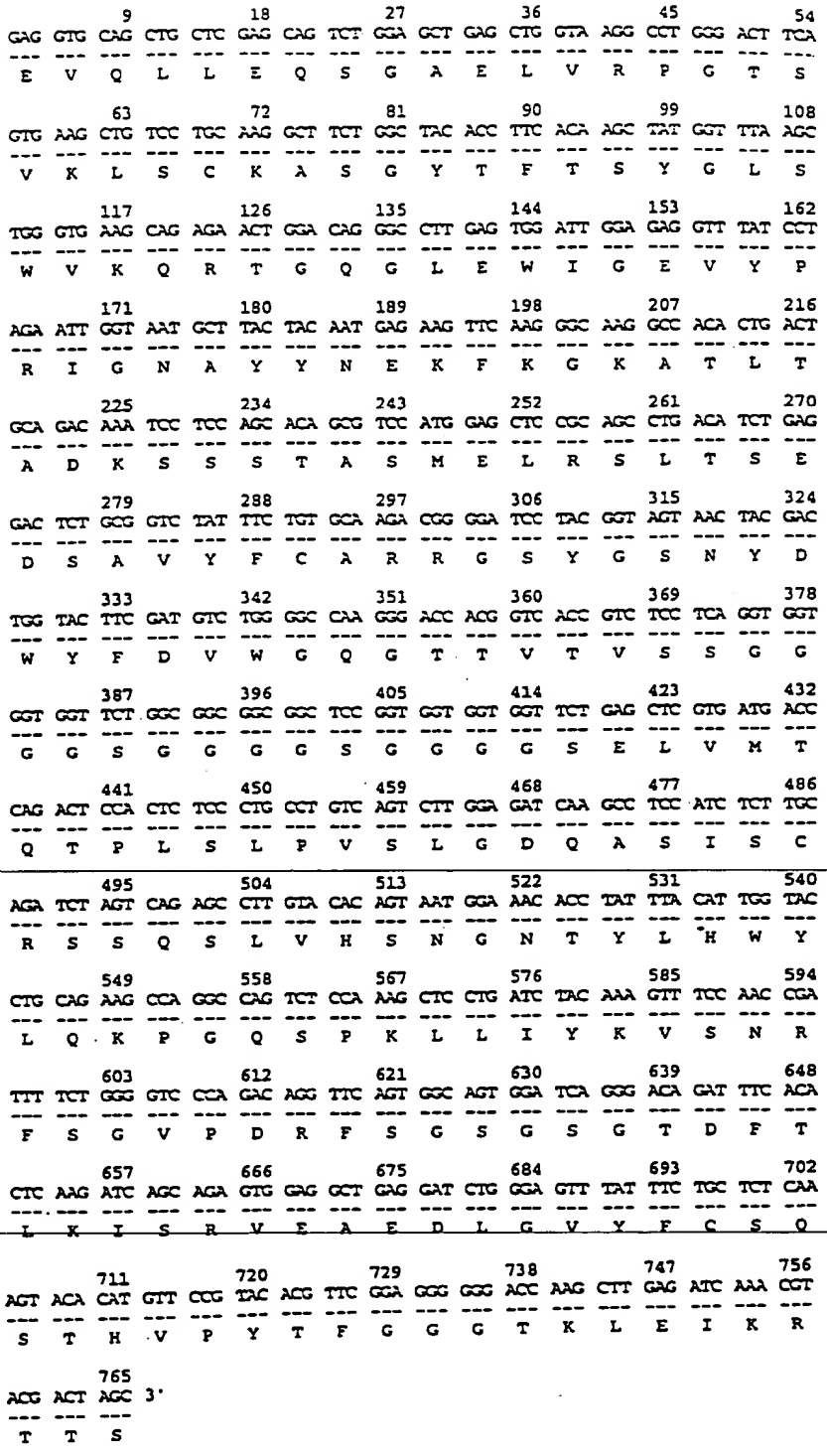
G G T K L E I K R. T T S

Figure 6.4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F S G V P D R F S G S G S G T D F T
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L K I S R V E A E D L G V Y F C S Q
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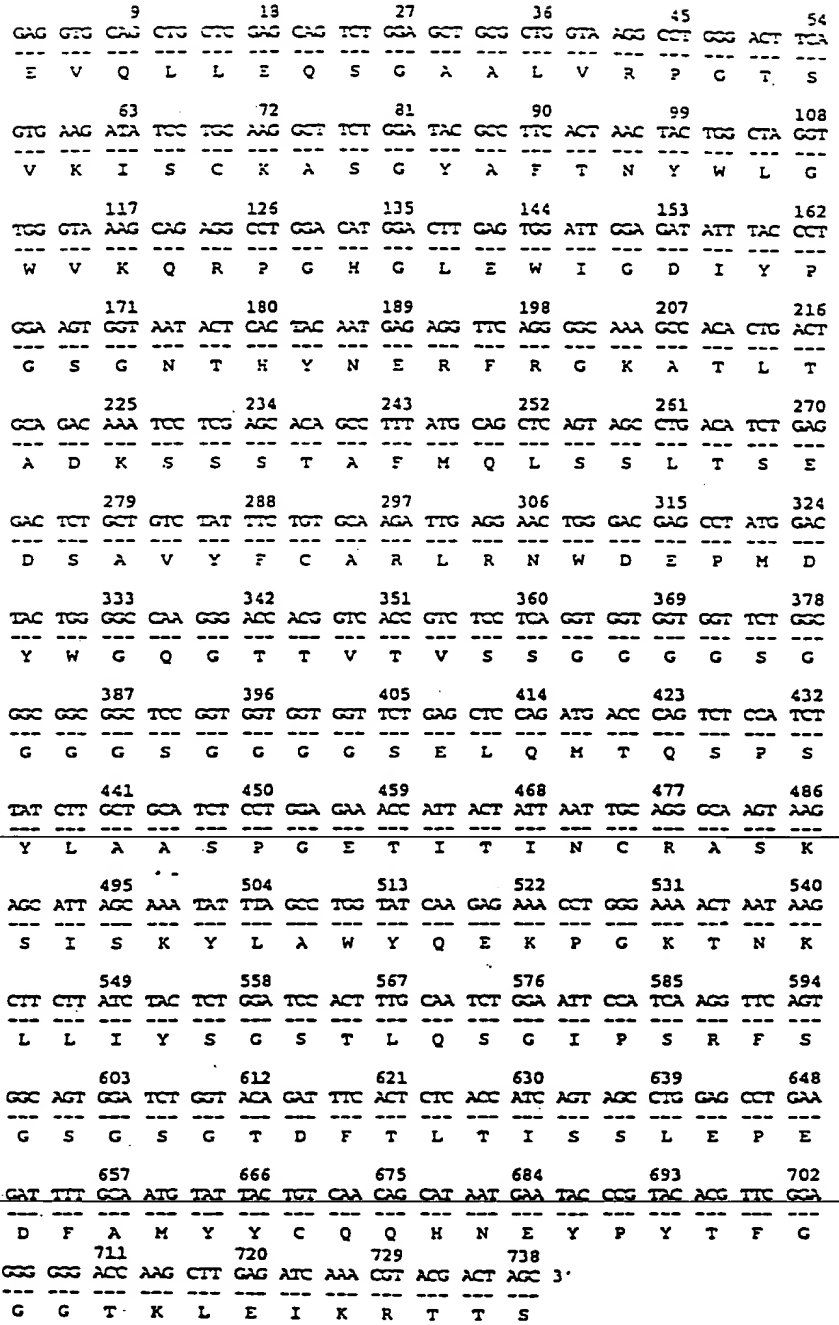
ACG ACT AGC 3'
T T S

Figure 6.5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D F A M Y Y C Q Q H N E Y P Y T F G
711 720 729 738
GGG GGG ACC AAG CTT GAG ATC AAA CGT ACG ACT AGC 3'
G G T K L E I K R T T S

Figure 6.6


```
                9                  18                  27                  36                  45                  54
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--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 E   V   Q   L   L   E   Q   S   G   A   E   L   V   R   P   G   T   S

               63                  72                  81                  90                  99                 108
GTG AAG ATA TCC TGC AAG GCT TCT GGA TAC GCC TTC ACT AAC TAC TGG CTA GGT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 V   K   I   S   C   K   A   S   G   Y   A   F   T   N   Y   W   L   G

              117                 126                 135                 144                 153                 162
TGG GTT AAG CAG AGG CCT GGA CAT GGA CTT GAA TGG GTT GGA GAT ATT TTC CCT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 W   V   K   Q   R   P   G   H   G   L   E   W   V   G   D   I   F   P

              171                 180                 189                 198                 207                 216
GGA AGT GGT AAT GCT CAC TAC AAT GAG AAG TTC AAG GGC AAA GCC ACA CTG ACT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 G   S   G   N   A   H   Y   N   E   K   F   K   G   K   A   T   L   T

              225                 234                 243                 252                 261                 270
GCA GAC AAG TCC TCG TAC ACA GCC TAT ATG CAG CTC AGT AGC CTG ACA TCT GAG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 A   D   K   S   S   Y   T   A   Y   M   Q   L   S   S   L   T   S   E

              279                 288                 297                 306                 315                 324
GAC TCT GCT GTC TAT TTC TGT GCA AGA TTG CGG AAC TGG GAC GAG GCT ATG GAC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 D   S   A   V   Y   F   C   A   R   L   R   N   W   D   E   A   M   D

              333                 342                 351                 360                 369                 378
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--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 Y   W   G   Q   G   T   T   V   T   V   S   S   G   G   G   G   S   G

              387                 396                 405                 414                 423                 432
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--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 G   G   G   S   G   G   G   G   S   E   L   V   M   T   Q   S   P   S

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--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 S   L   S   V   S   A   G   E   K   V   T   M   S   C   K   S   S   Q

              495                 504                 513                 522                 531                 540
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--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 S   L   L   N   S   G   N   Q   K   N   Y   L   A   W   Y   Q   Q   K

              549                 558                 567                 576                 585                 594
CCA GGG CAG CCT CCT AAA CTG TTG ATC TAC GGG GCA TCC ACT AGG GAA TCT GGG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 P   G   Q   P   P   K   L   L   I   Y   G   A   S   T   R   E   S   G

              603                 612                 621                 630                 639                 648
GTC CCT GAT CGC TTC ACA GGC AGT GGA TCT GGA ACA GAT TTC ACT CTC ACC ATC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 V   P   D   R   F   T   G   S   G   S   G   T   D   F   T   L   T   I

              657                 666                 675                 684                 693                 702
AGC AGT GTG CAG GCT GAA GAC CTG GCA GTT TAT TAC TGT CAG AAT GAT TAT AGT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 S   S   V   Q   A   E   D   L   A   V   Y   Y   C   Q   N   D   Y   S

              711                 720                 729                 738                 747                 756
TAT CCG TAC ACG TTC GGA GGG GGG ACC AAG CTT GAG ATC AAA CGT ACG ACT AGC 3'
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
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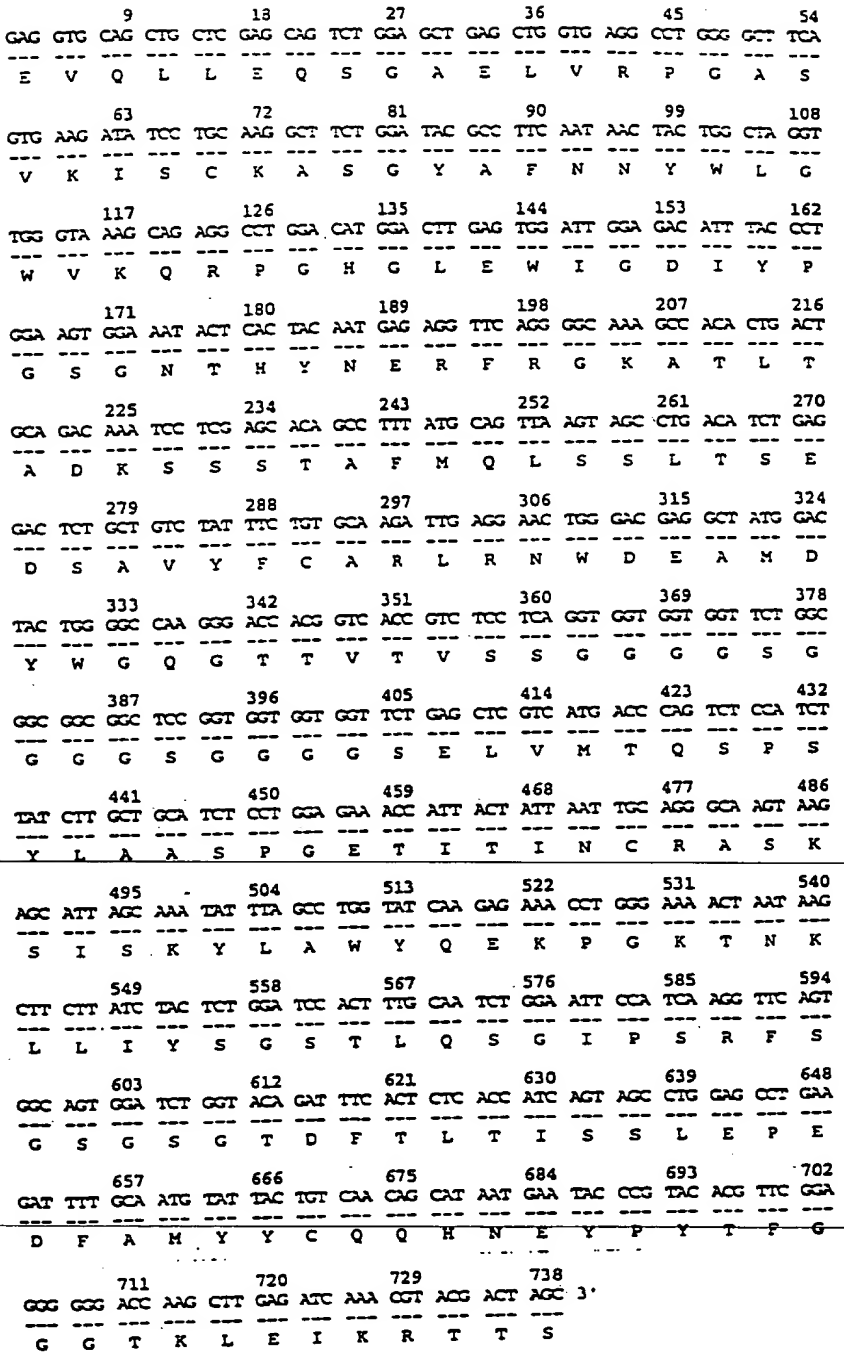
Figure 6.7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L L I Y S G S T L Q S G I P S R F S
603 612 621 630 639 648
GGC AGT GGA TCT GGT ACA GAT TTC ACT CTC ACC ATC AGT AGC CTG GAG CCT GAA
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D F A M Y Y C Q Q H N E Y P Y T F G
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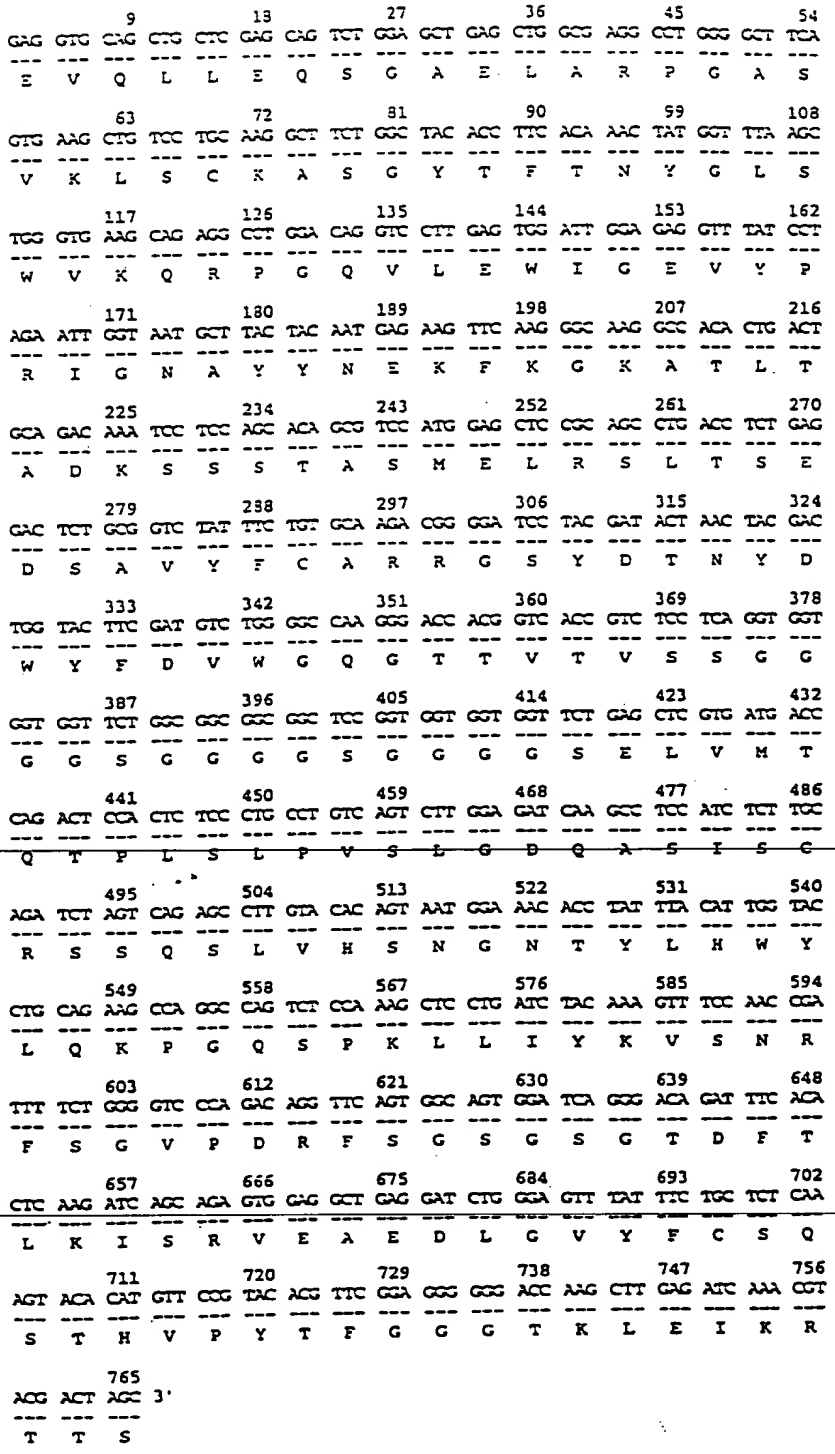
G G T K L E I K R T T S

Figure 6.8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GTG AAG CTG TCC TGC AAG GCT TCT GGC TAC ACC TTC ACA AAC TAT GGT TTA AGC
V K L S C K A S G Y T F T N Y G L S
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R I G N A Y Y N E K F K G K A T L T
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Figure 6.9

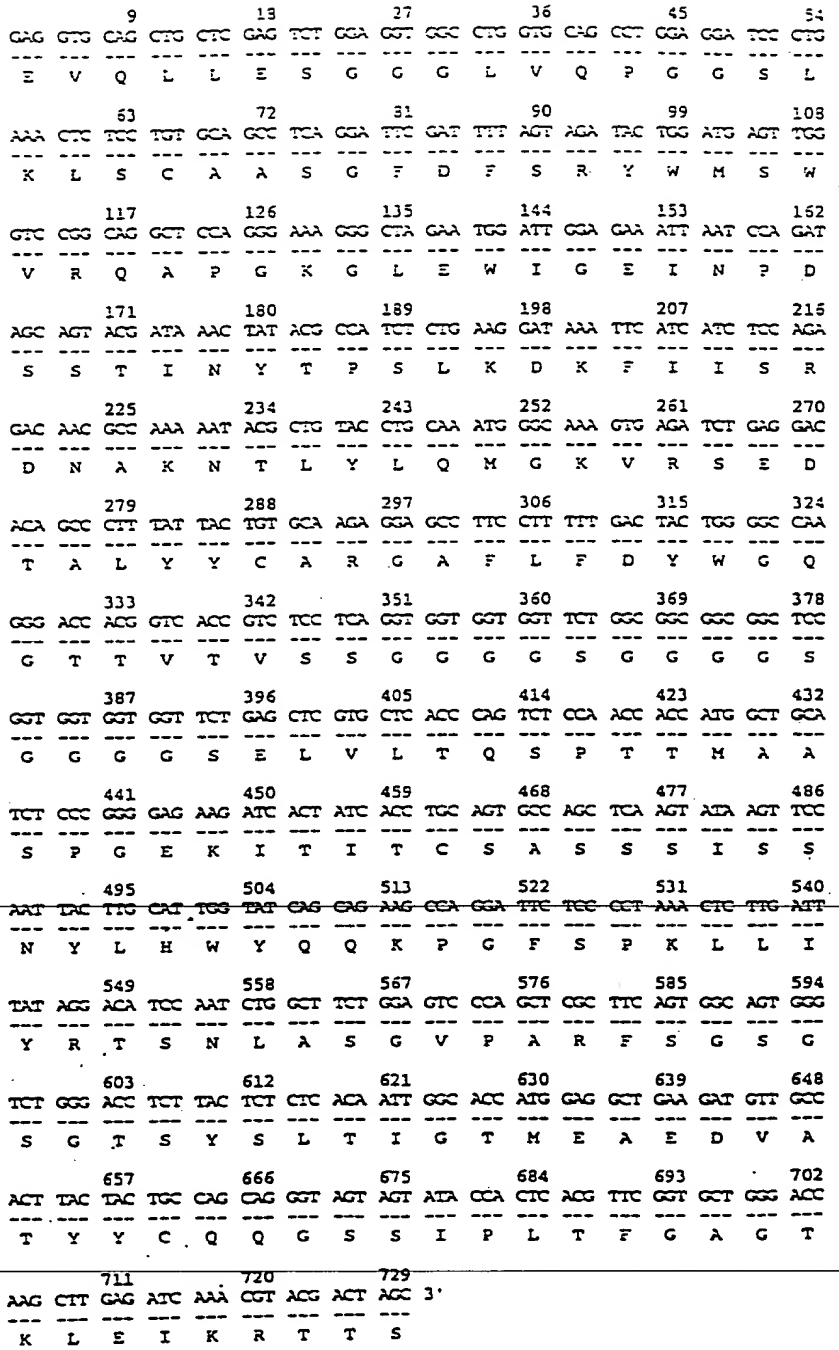
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Figure 6.10

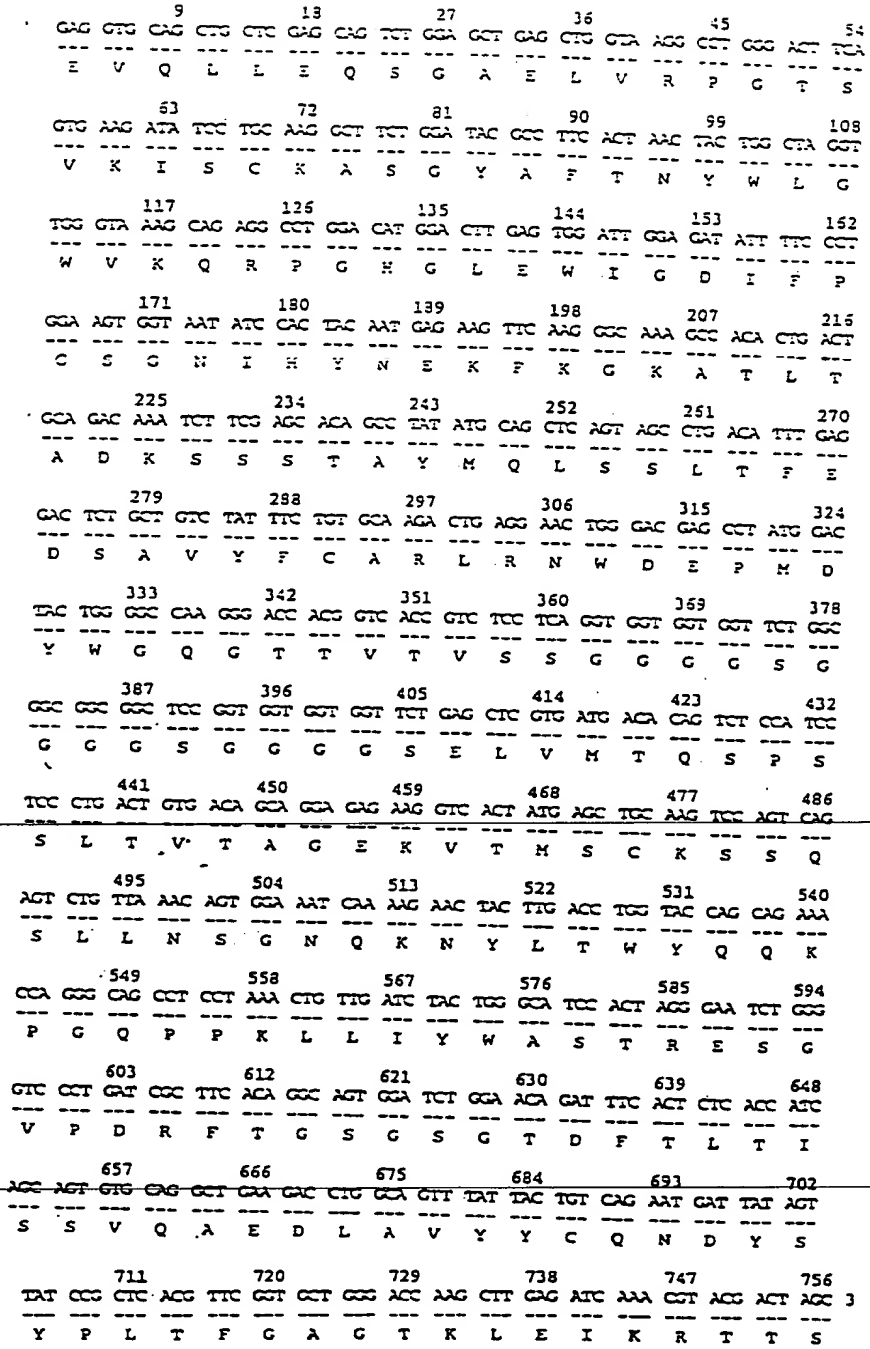
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Figure 7

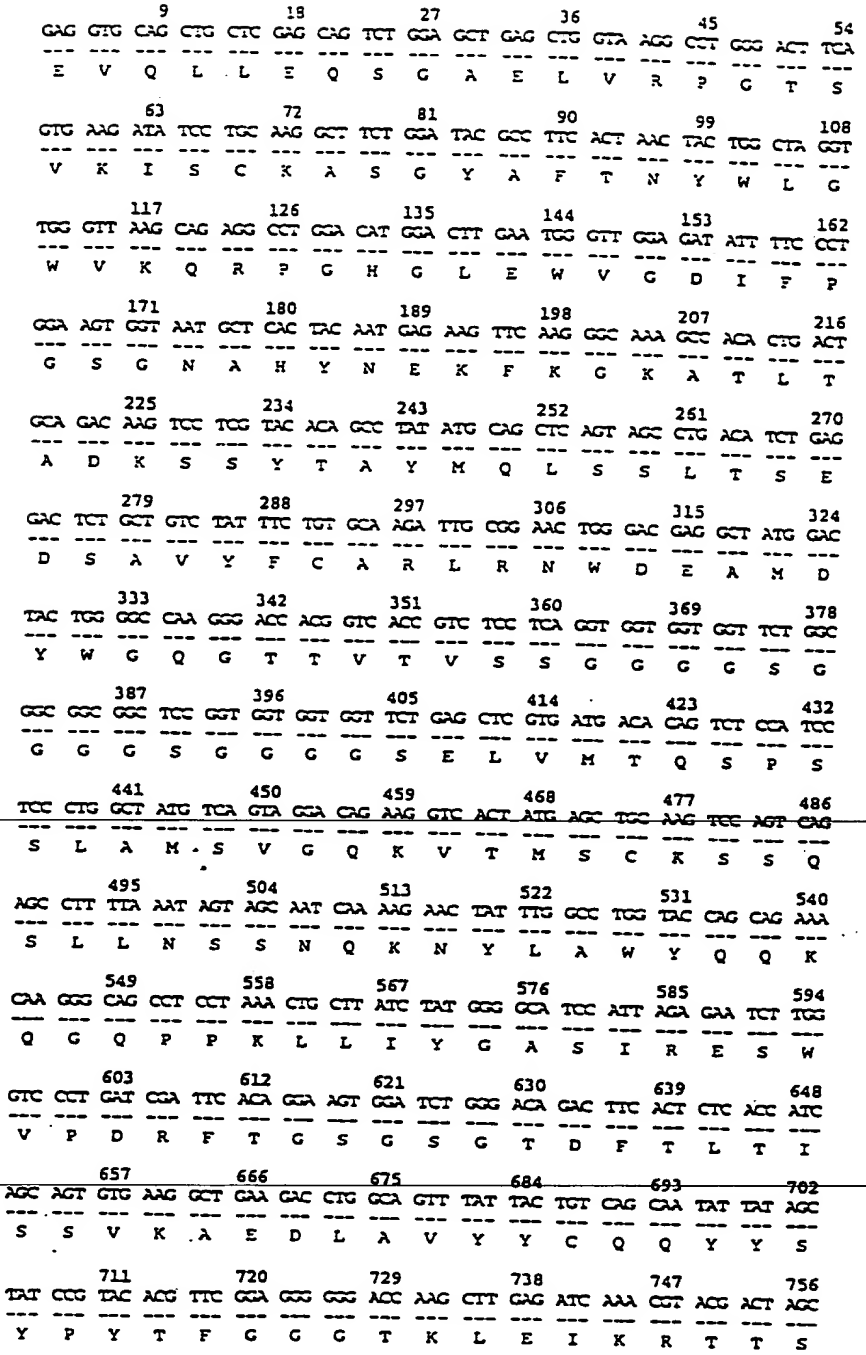
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Figure 8.1

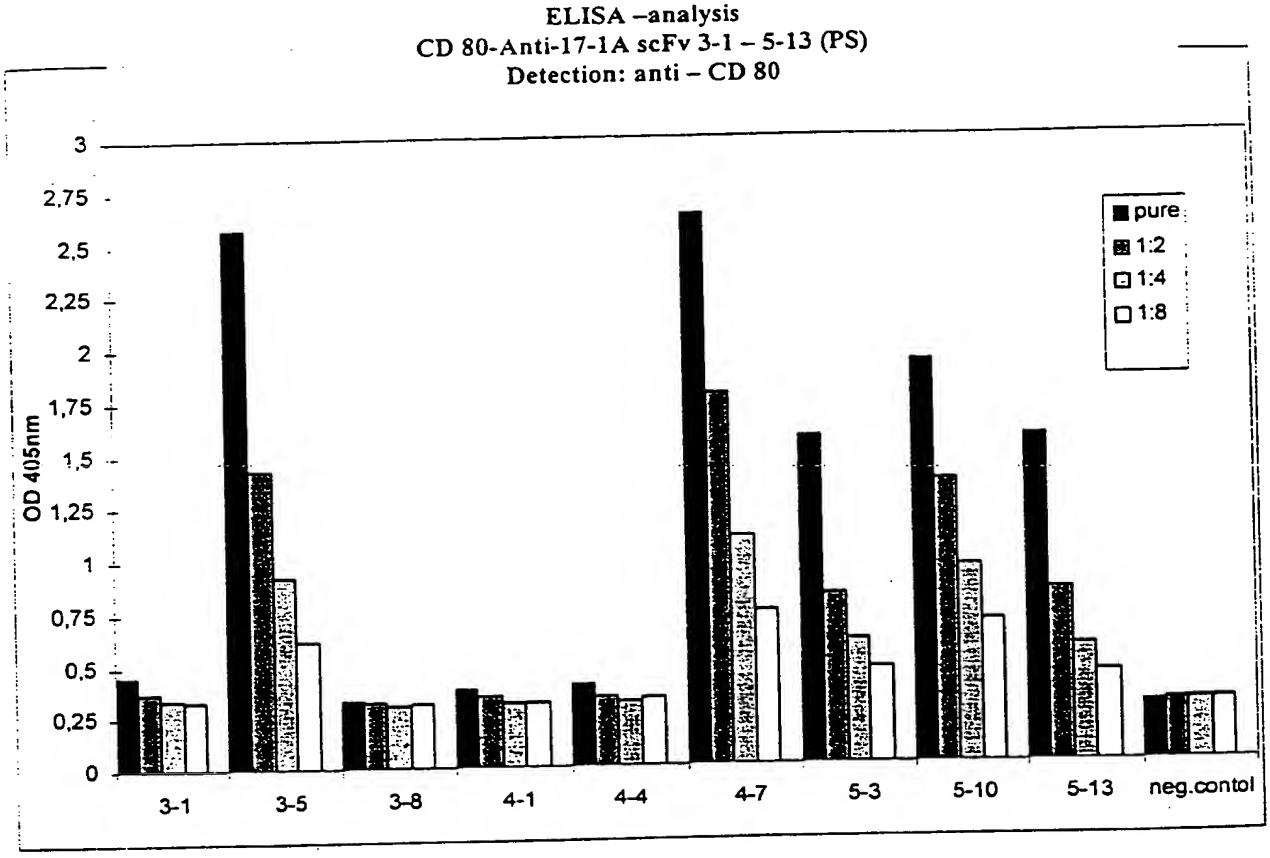

Figure 8.2

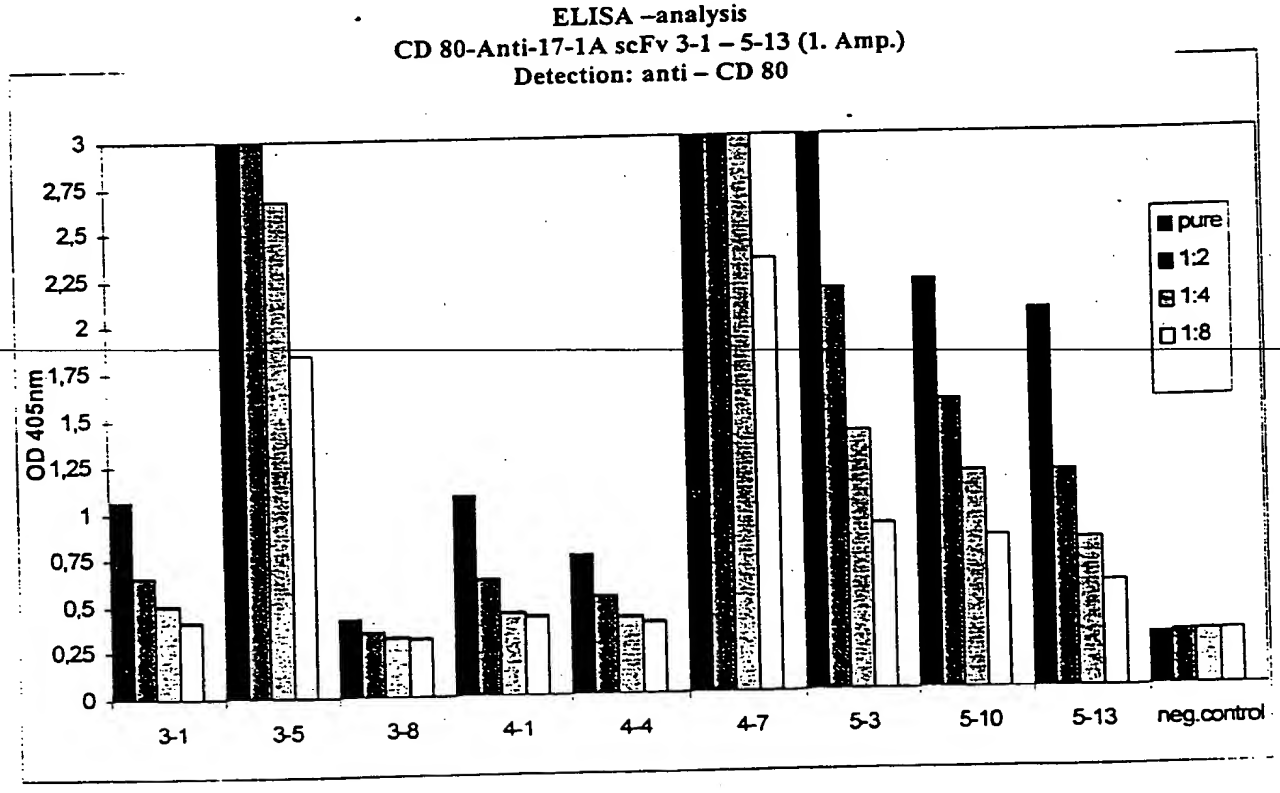

Figure 8.3

ELISA –analysis
CD 80-VD4.5Vk8 scFv (PS) and CD 80-Mach scFv (PS)
Detection: anti – CD 80

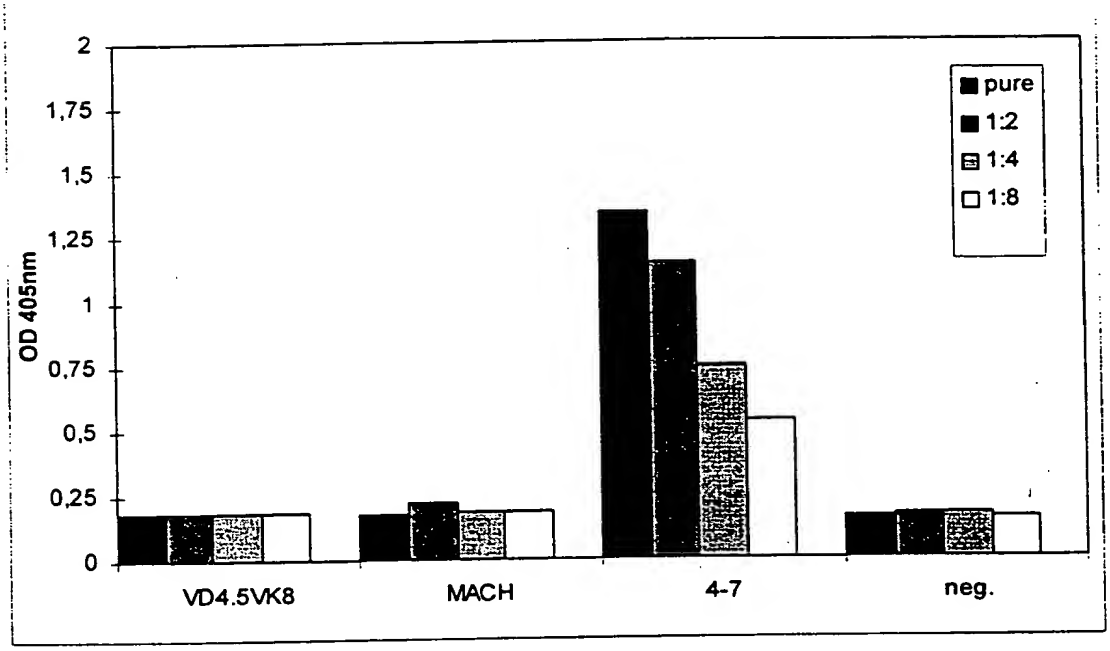

Figure 8.4

ELISA –analysis
CD 80-VD4.5Vk8 scFv (1. Amp.) and CD 80-Mach scFv (1. Amp.)
Detection: anti – CD 80

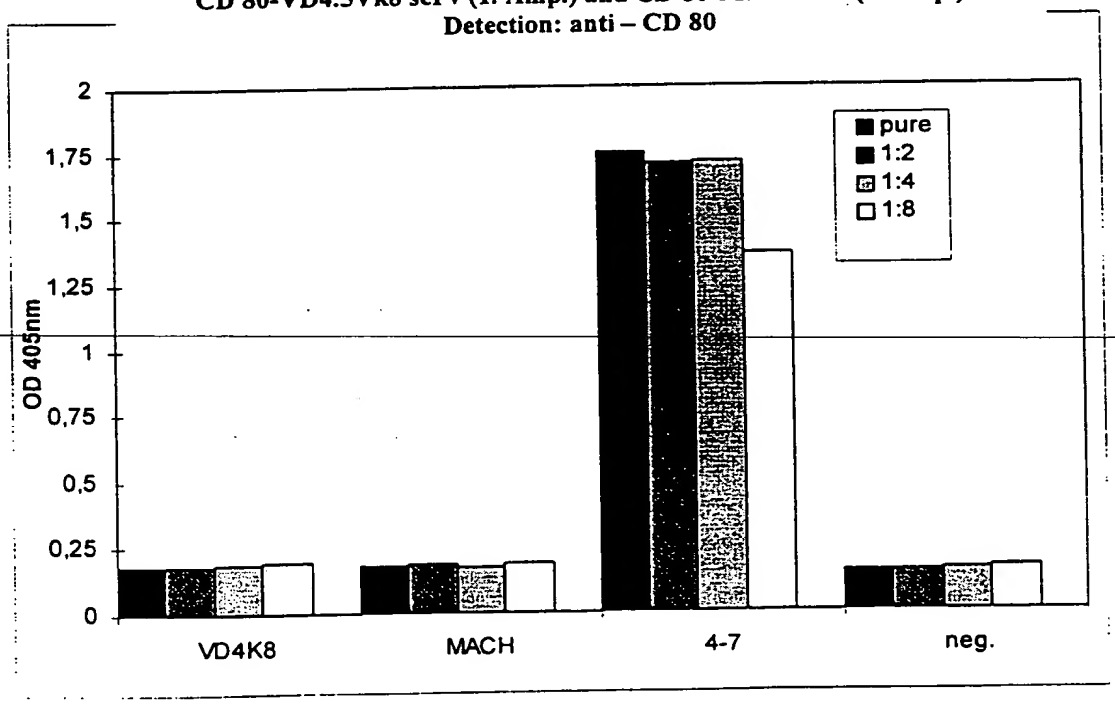

Figure 9.1

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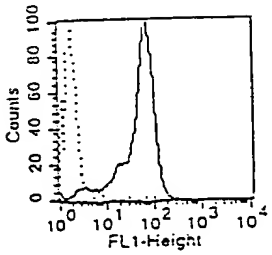

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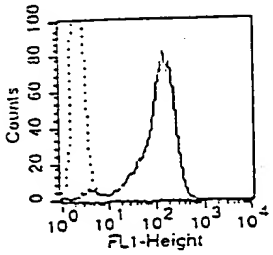

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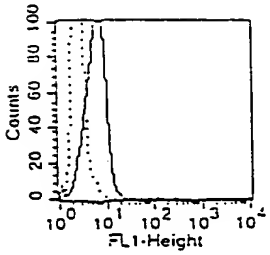

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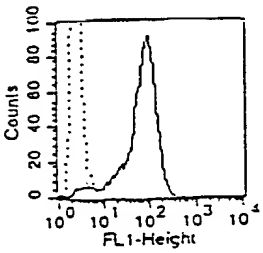

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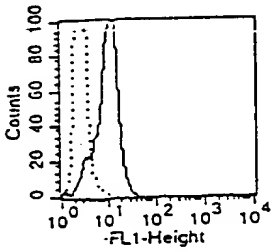

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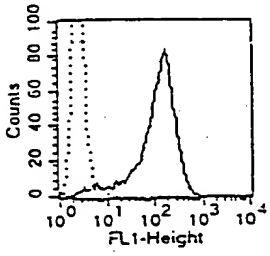

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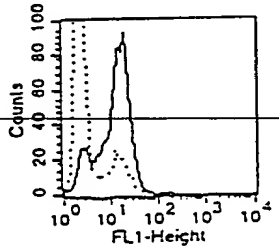

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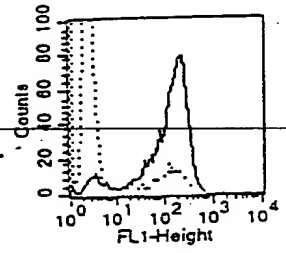

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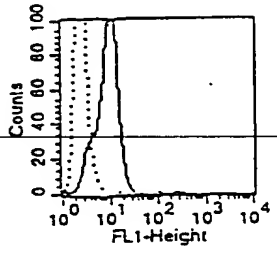

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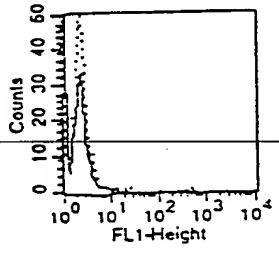
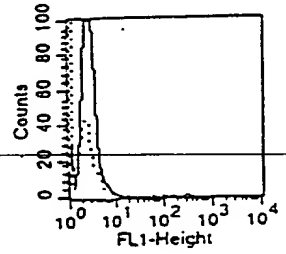
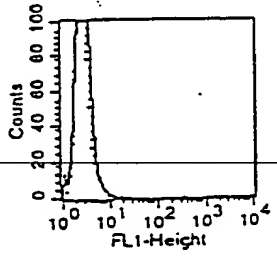


MACH



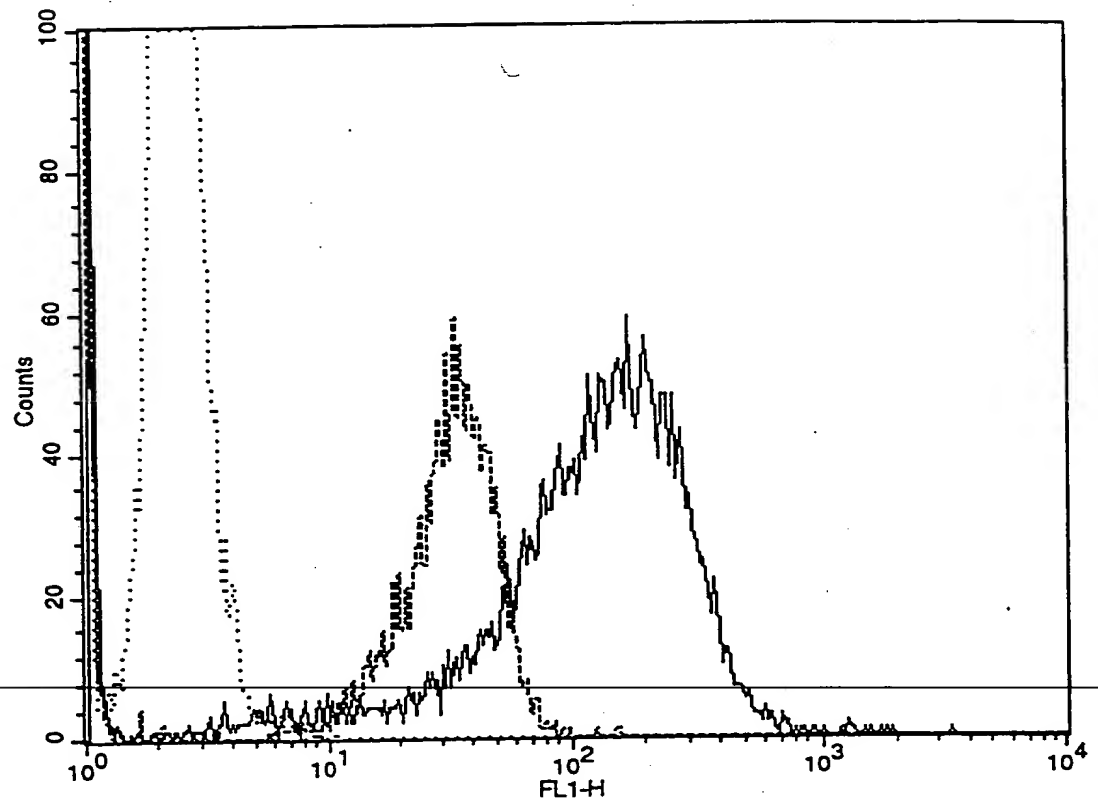
neg.Co



..... untransfected CHO cells

_____ 17-1A transfected CHO cells

Figure 9.2



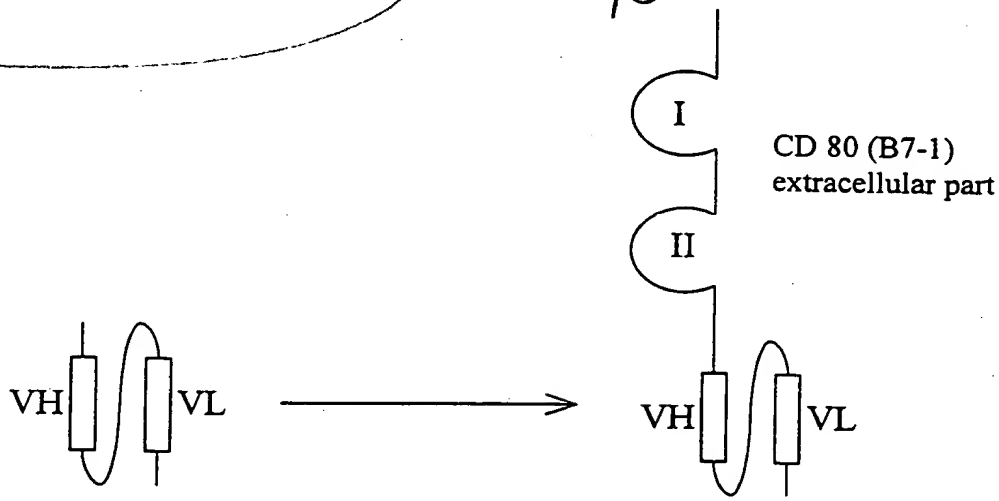
_____ M79 on 17-1A transfected CHO cells
..... M79 on untransfected CHO cells
----- M79 on KATO cells

Figure 10

1) The Conventional approach

Randomly selected antigen–specific VH/VL-pairs that bind to their antigen as free or N-terminally located scFv-fragments or as whole antibody molecules

Frequent loss of antigen binding after fusion of another protein domain to the N-terminus of the scFv-fragment



2) The method of invention

VH/VL–pairs selected by the method of the invention

High frequency of antigen binding after fusion of another protein domain to the N-terminus of the scFv-fragment

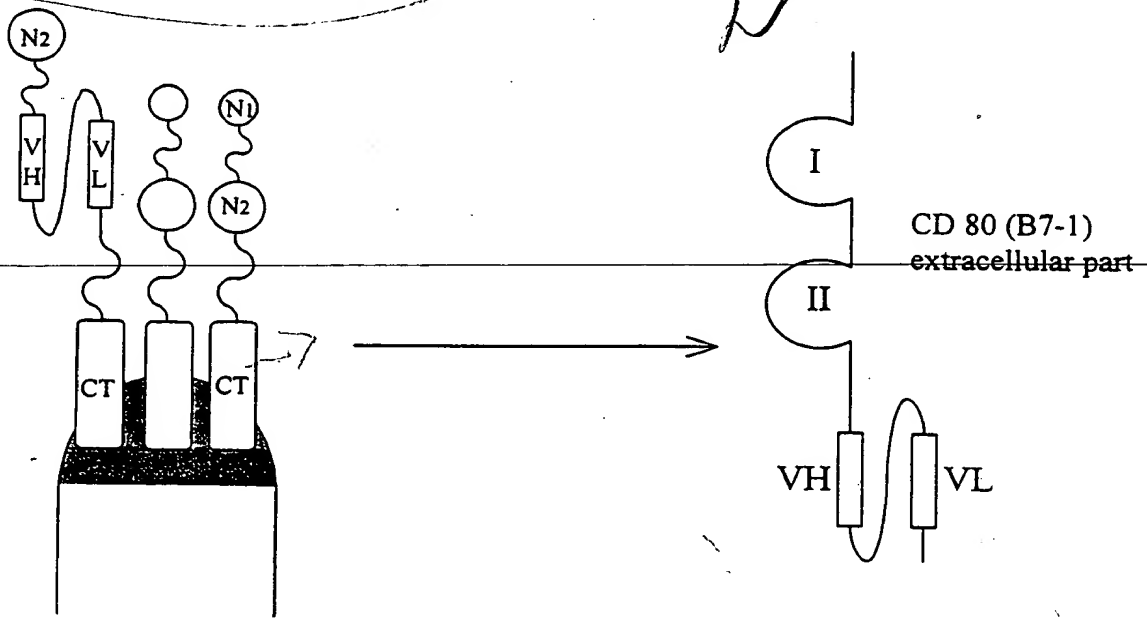
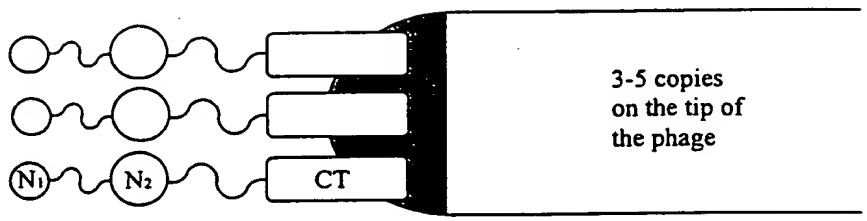
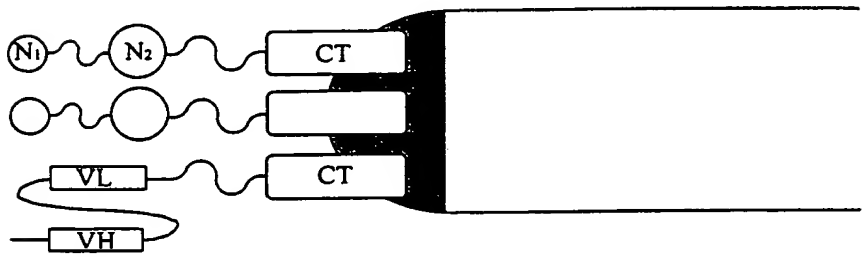


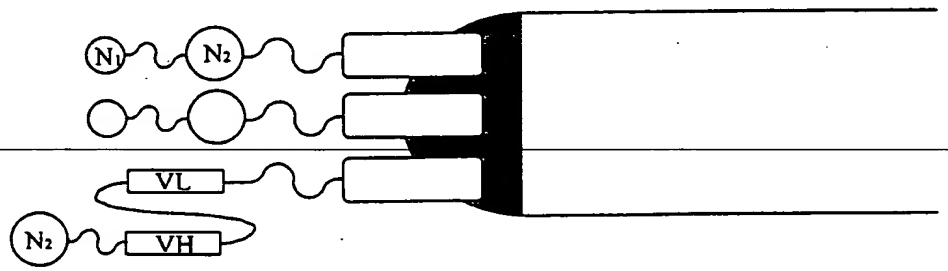
Figure 11



Wildtype phage



Conventional
display phage



display phage
according to
the method of
the invention